TUBULE DENSITY, MOISTURE CONTENT AND MECHANICAL PROPERTIES OF DONKEY HOOF HORN

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DECLARATION OF ORIGINALITY

This is to certify that I am responsible for the work in this thesis. The work is my own unless otherwise acknowledged. Neither this thesis nor the original work reported therein has been submitted to this nor any other institution for a higher degree. The work was carried out in the Faculty of Applied Sciences at De Montfort University, Leicester and was supervised by Pro. R. J. Latham and Lt Col. J.D. Reilly.

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ABSTRACT

This study established new protocols for the collection, storage and preparation of donkey hoof horn samples prior to their subsequent analysis. Using these methods, normal values for moisture content, tubule density and mechanical properties for donkey hoof horn were established. Novel methods for investigating regional differences in these hoof horn parameters were also validated in this study.

The protocols for, and calculations used in, establishing hoof wall moisture content were substantially reviewed in this study. There were significant differences between moisture contents of donkey hoof horn ascertained using different drying techniques. One technique, namely desiccation over phosphorus pentoxide, was subsequently chosen to be the most appropriate method for dehydration of hoof horn for analyses of moisture content. The moisture content of donkey hoof horn of 33% was found to be significantly higher ($p<0.01$) than the 26% shown for horse hoof horn.

Donkey and horse hoof horn are, *in vivo,* close to full hydration levels. This is a different situation to that previously believed to exist for the horse and may have important implications for the management of donkey hoof horn and the types of disease to which it is subject.

The sorption and desorption isotherms for donkey hoof horn samples equilibrated over different saturated salt solutions showed hysteresis. It is thus necessary to know whether the moisture content of donkey horn samples following equilibration in a particular relative humidity environment has been achieved by sorption or desorption. The sorption and desorption isotherms for donkey hoof horn showed similar results to other keratinised tissues such as wool and head horn.

Analyses were carried out on full hoof wall depth samples and also on partial hoof wall depth samples. These latter samples were known as zones, with zone 1 being the most outer part of the *Stratum medium* and zone 4 being the most inner part. There was a

dorso-palmar increase in moisture content. There were significant differences in moisture content between all zones except between zone 3 and zone 4 for donkey hoof horn.

A three zoned pattern of distribution of hoof horn tubules across the dorso-palmar depth of the hoof wall has been shown to exist for donkey hoof horn, compared to the four zoned pattern that is already known to exist for pony and horse hoof horn. These differences may reflect important differences in the mechanisms of hoof function. There was a positive correlation (0.92) between hoof wall depth and animal bodyweight ($p<0.001$). A regression analysis resulted in an R^2 value of 85%.

Donkey hoof horn was shown to display Hookean properties during flexural three point bending. There was a significant inverse relationship between moisture content and mechanical properties of donkey hoof horn. Indeed, 96% of the variation in modulus was attributed to the effect of moisture content.

The mechanical properties of horse hoof horn were thought to vary across the hoof wall. However, for donkey hoof horn, the use of a consistent level of hydration indicated that the mechanical properties do not vary across the four zones tested.

There was a positive correlation (0.64) between tubule density and the moduli of samples when tested at full hydration ($p<0.05$), together with a positive correlation (0.60) between tubule density and the hydrated regain of samples equilibrated at 75% relative humidity ($p<0.05$).

This study provided objective measurement of moisture content, tubule density and mechanical properties of donkey hoof horn. Assessment of both clinical hoof problems, and the results of manipulation of nutrition or management of animals on hoof condition, can now be carried out objectively.

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ABBREVIATIONS, SYMBOLS AND GLOSSARY OF TERMS USED IN THE THESIS

*	Significant difference at 95% confidence level
**	Significant difference at 99% confidence level
ANOVA	Analysis of Variance
b	Width of Hoof Wall
BWT	Bodyweight
CV	Coefficient of Variation
d	Depth of Hoof Wall
DW	Dry Weight
E	Modulus of Elasticity
ε	Strain
F	Female
FMC_F	Fresh Moisture Content as a Percentage of Fresh or *in vivo* Weight
FMC_D	Moisture regain
FW	Fresh Weight
g	Grammes
GAGs	Glycosaminoglycans
HMC	Hydrated Moisture Content as a percentage of Wet Weight
HMC_D	Hydrated regain
HWD	Hoof wall depth
HWH	Hoof wall height
I	Moment of Inertia
IFs	Intermediate Filaments
IFAPs	Intermediate Filament Associated Proteins
kg	Kilogram
l	Sample length
LF	Left Fore
M	Male
MC	Moisture Content
MDC	Midline Dead Centre
mg	milligramme
mm	millimetres
MPa	Mega Pascals
NS	Not significant
P_2O_5	Phosphorus Pentoxide
Poly	Polynomial
RH	Relative Humidity
R^2	R square value
S	Span
SB	*Stratum basale*
SD	Standard Deviation
SG	*Stratum germinativum*
SM	*Stratum medium*
SMZA	*Stratum medium zona alba*

SS	*Stratum spinosum*
TD	Tubule Density
με	Micro strain
WW	Wet Weight
Z1 ... 4	Zone 1 ... 4

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1. INTRODUCTION

1.1 General Introduction

Horses and other animals are reliant on the hoof horn capsule for effective locomotion (Reilly and Kempson 1992). The hoof is a highly keratinized, avascular, epidermal structure surrounding, supporting and protecting underlying sensitive tissues from friction, extremes of heat and cold, infection and dehydration or water absorption (Pollitt 1992). In this way, it is remarkable in that it affords protection from major stresses, both chemical and physical, to which the hoof is exposed (Cope *et al* 1998). The hoof must also be able to withstand concussion and be able to resist abrasion and wear. Defects in the hoof horn reduce its functional integrity and are a major cause of reduced vitality and suffering in the species concerned (Kempson 1990). The well known term "no hoof, no horse" may be applied equally to all equines.

Hoof management for all equines has tended to be a skill rather than a science and throughout the general hoof literature there is a paucity of detailed information about hoof management that has been supported by scientific research. Weaver (1981) recognised the difficulties in measuring physical hoof qualities together with the difficulties in the comparison of results owing to a lack of standardised methods. Scientific research into hoof horn has also been hampered by the absence of objective and controlled methods of assessment (Cuddeford 1991; Reilly 1995; Slater and Hood 1997). Presumably this is partly due to the high costs involved in keeping equines (Reilly 1995) and partly due to limited access to sufficiently large numbers of equines that are managed under similar conditions (Josseck *et al* 1995; Reilly 1995).

Despite common misconceptions, the donkey (*Equus asinus*) is a separate species to the horse (*Equus caballus*). Donkeys are generally used as beasts of burden in the third world (Varshney and Gupta 1994) or used as pleasure animals in the developed world (Reilly 1997). Donkey hoof has been treated the same as that of other equines despite there being gross anatomical differences between the hooves of donkeys and

horses as shown by different authors (Lungwitz and Adams 1913; Hifny and Misk 1983; Fowler 1995; Reilly 1997).

Information on specific problems of the donkey hoof is also scarce, though Comben *et al* (1983) referred to little evidence of crumbling or disintegration of the lower edges of the hoof walls compared to horses. This may have been because donkeys are generally not shod. Comben *et al* (1983) did, however, find cases showing wall and sole separation, white line lesions and soft wall horn. However, it is believed that donkeys in the UK tend to have more hoof problems than those living in their native hot, arid climates with little food (Trawford, A. personal communication).

The majority of donkeys in the UK are not shod and, indeed, do not need to be shod as horn production is sufficient in quantity to cope with work, even on metalled roads (Fowler 1995).

The large worldwide population of approximately 43 million donkeys compared with 58 million horses (FAO 2001) also appears to have been overlooked as there is very little information available on donkey hoof horn.

The "quality" of hoof horn is referred to continuously but "quality" has yet to be quantified in objective terms to thus avoid the very subjective assessments presently used. The "norm" of different hoof horn characteristics for particular populations can be assessed. The manipulation of other factors, such as environment, can then be assessed quantitatively. The manufacturers of many dietary supplements claim they improve the quality of hoof horn. If changes are assessed quantitatively then their claims could be substantiated. This provides opportunities to improve the management of animals, thus aiding in their welfare.

Despite the obvious influence of the hoof for locomotion and welfare for all hooved animals, there is still little scientific research available on important factors such as the physiological, anatomical and mechanical properties of equine hoof horn (Reilly 1999). Analyses of these characteristics will also contribute to the knowledge of the

interactions of the structure and function of hoof horn. Therefore, in order to make progress in the field of hoof research, it is necessary to employ objective methods to fully describe the normal healthy hoof (Reilly 1995).

The aim of this thesis is given in section 1.13. However, the specific objectives of the work, in order to address the overall aim were:

- To establish protocols using donkey hoof horn samples available from clippings in order:

    - To characterise tubule density;
    - To determine moisture content;
    - To investigate mechanical properties.

- To establish the inter-relationships of these three parameters.

1.2 Anatomy of the Equine Hoof

In 1983 Hifny and Misk reported that there was very little published work on donkey hoof and this position has still not changed. The majority of this section on anatomy has therefore been taken from the literature on horse hoof. Differences between the hoof anatomy of the two species is, however, considered later in Table 1.1.

The hoof is a highly evolved locomotor organ of epidermal origin which is avascular and devoid of nerve endings (Sisson and Grossman 1953), thus making it insensitive. It has a complex three dimensional structure and consists of a horny capsule which encases bones, joints, blood vessels, nerves, ligaments, tendons and bursae, and connective and adipose tissues (Kainer 1989). The hoof horn capsule and its contents are collectively known as the 'foot' (Smith 1921).

1.2.1 Dermis or Corium

The dermis or corium contains the nerves and blood vessels which supply the capsule with its nutrients and is composed of collagenous material rich in elastic fibres. The corium forms a continuous layer and is divided into the perioplic, coronary, laminar, solear and frog coria (Sack and Habel 1977). The coria are shaped into papillae, except for the laminar corium, which is in a lamellar form (Dyce *et al* 1987).

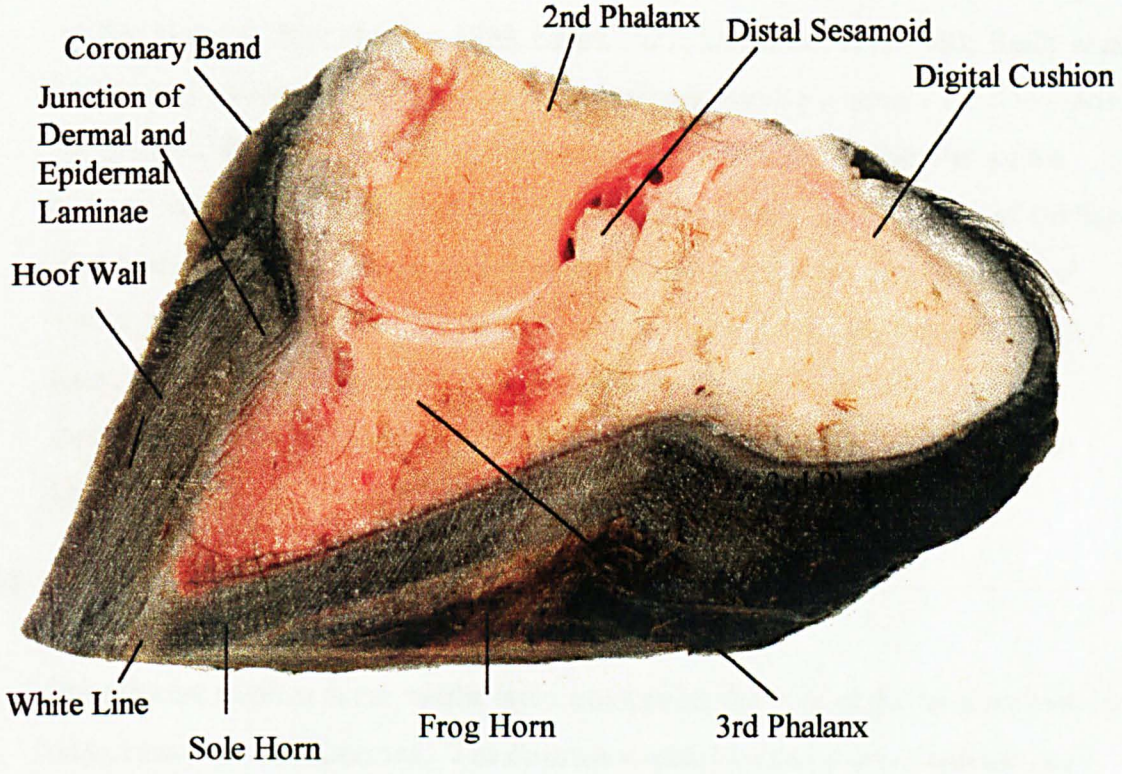
The epidermal components of the hoof are generally divided into the wall, sole, frog and white line (Figure 1.1).

1.2.2 Wall

The hoof wall is the part of the hoof visible in the standing animal (Pollitt 1992). The outer part of the hoof is the *Stratum corneum*. The major part of the wall grows distally from the coronary band which is at the junction of the wall and the skin (Pollitt 1990). The border between the wall and the ground is known as the bearing border (Sisson and Grossman 1953). The wall is arbitrarily divided into the toe, medial and lateral quarters, medial and lateral heels and it is reflected at the bearing

border at the heels to form the bars (Stump 1967). In horses, the wall is thickest at the toe and becomes thinner towards the heels (Kainer 1989). For horses, the rate of horn growth is approximately 8-10 mm per month so that 9-12 months are needed for wall growth from proximal to distal border (Stump 1967; Schummer *et al* 1981). Assessment of growth rate provides a means of quantitative analysis of hoof horn and has been used as a method of assessing the effect of nutritional supplementation on the growth of horse hoof horn (*e.g.* Reilly *et al* 1998a, Reilly *et al* 2002a).

Figure 1.1 - Sagittal Section of Donkey Hoof



The wall consists of three layers:

1 *Stratum externum*

2 *Stratum medium*

3 *Stratum internum*

1.2.3 *Stratum externum*

The *Stratum externum* is a thin layer of tubular horn covering the surface of the wall. The proximal part is the soft and *non*-pigmented periople overlying the proximal part of the hoof wall and expands caudally over the bulbs of the heels and approaches the ground surface of the foot and is produced by the perioplic corium (Pollitt 1992). The periople is believed to prevent moisture evaporation from the hoof (Fleming 1871a; Mettam 1896; Hunting 1899; Smith 1921; Schummer *et al* 1981; Reilly *et al* 1998a) and tends to be worn away in older animals, leaving a narrow band towards the proximal border. The lipid fraction of the periople is higher than that of the *Stratum medium* and is believed to offer the barrier to moisture (Reilly *et al* 1998a). This lipid fraction can also be altered by nutritional supplementation (Reilly *et al* 1998a; Reilly *et al* 2002b). The periople is worn away distally leaving a thin, flat layer, the *Stratum tectorium* which is composed of lipid and may also reduce evaporative water loss from the hoof (Leach 1980; Schummer *et al* 1981; Pollitt 1992).

1.2.4 *Stratum medium*

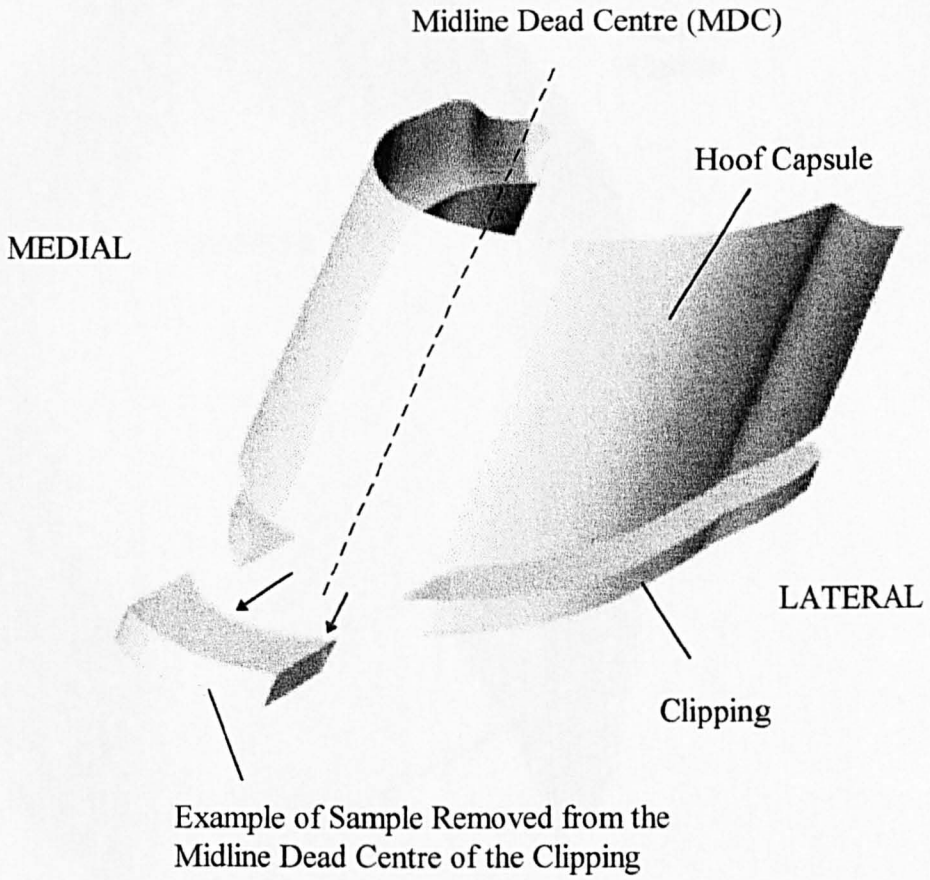
The *Stratum medium* is the middle layer, comprising the bulk of the hoof wall which may or may not be pigmented. The *Stratum medium* is composed of tubular and intertubular horn growing distally from the coronary band (Stump 1967) which is found at the most proximal edge of the hoof wall. The *Stratum medium* is the subject of this thesis. The horn tubules are produced by the germinal epidermis (*Stratum germinativum*) covering the long papillae of the coronary corium. The papillae are orientated parallel to the long axis of the hoof wall causing the horn tubules to have the same orientation. Intertubular horn is formed between the

papillae (Stump 1967; Pollitt 1992). Both the tubular and intertubular horn are insensitive (Leach 1980). Further information on tubular and intertubular horn formation is provided in section 1.3.

The midline dead centre (MDC) of the hoof capsule, and therefore also of the *Stratum medium*, has been defined by Reilly *et al* (1996) (Figure 1.2). This area has been used as a specific sampling reference point (Reilly *et al* 1996, 1998b).

The most distal part of the *Stratum medium* is also removed during routine farriery and is known as a clipping (Figure 1.2 and Figure 1.3).

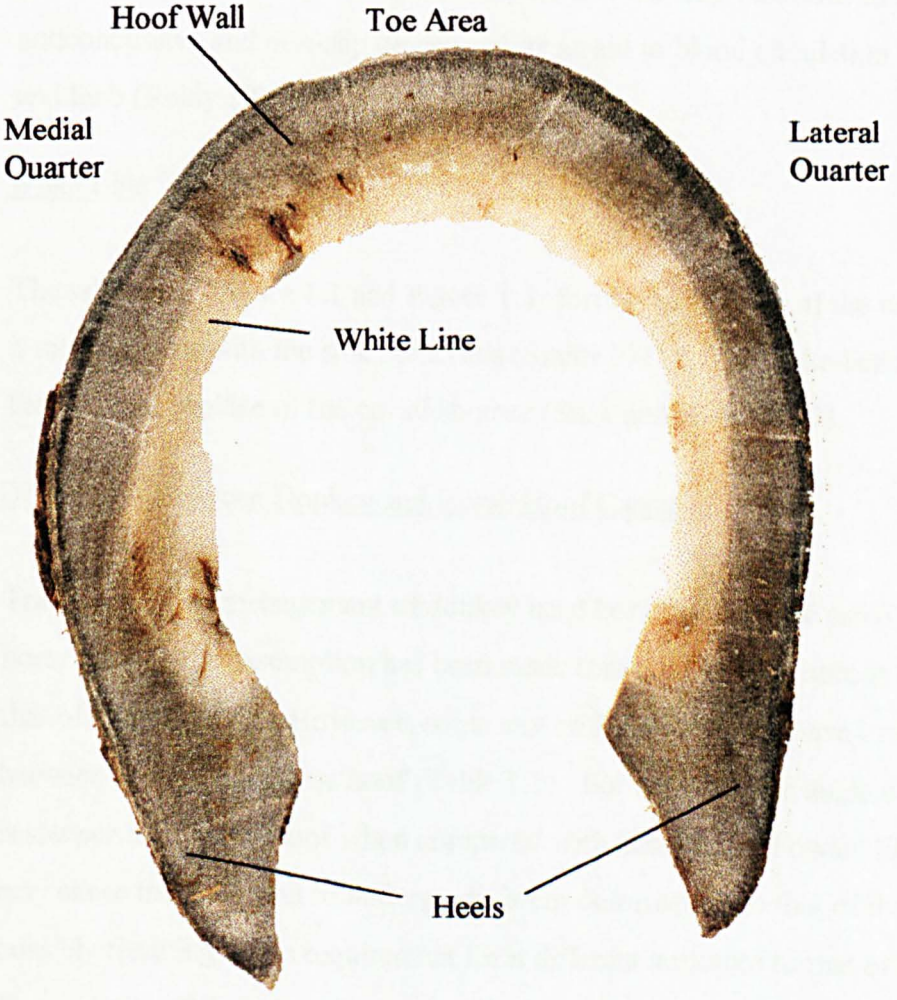
Figure 1.2 - Position of Midline Dead Centre Sample Site and Clipping on Left Fore Hoof Capsule



1.2.5 *Stratum internum*

The *Stratum internum* is a *non*-pigmented internal layer which, in horses, comprises approximately 600 keratinised primary epidermal laminae with secondary epidermal laminae (Stump 1967). Each keratinised primary epidermal lamina bears 100-150 *non*-keratinised secondary epidermal laminae (Pollitt 1992) which dovetail with the primary and secondary dermal laminae from the laminar corium. The number of laminae is believed to be lower for donkey hoof but numerical data were not provided (Hifny and Misk 1983). This intricate arrangement of laminae provides a large surface area for attachment and suspension of the third phalanx within the horny capsule (Stump 1967) and therefore ultimately suspends the whole animal.

Figure 1.3 - An Example of a Donkey Hoof Clipping from a Left Fore Limb



1.2.6 Sole

The sole provides the base of the hoof and is produced from the solear corium overlying the third phalanx. It is also composed of tubular and intertubular horn. The sole is said to be concave under natural conditions and therefore does not bear weight and is said to shed or 'flake off' its outermost layers. There is still debate as to whether the sole of the donkey hoof should bear weight (Reilly 1997).

1.2.7 Frog

The frog is a wedge-shaped mass of horn with a rubbery consistency, situated at the heels and pointing forward into the sole of the foot (Smith 1921). The frog is produced from the frog corium. It may be that the frog functions as an anticoncussive and *non*-slip device and as an aid to blood circulation within the foot and limb (Reilly 1997).

1.2.8 White Line

The white line (Figure 1.1 and Figure 1.3) forms the junction of the wall and sole as it interdigitates with the sole epidermis (Smith 1921). The white line is formed by the terminal papillae of the corial laminae (Sack and Habel 1977).

1.2.9 Differences Between Donkey and Horse Hoof Capsules

Traditionally the management of donkey hoof horn has been the same as that for horse hoof as the assumption has been made that donkey hoof horn is the same as that of other equines. However, some anatomical differences have been noted between donkey and horse hoof (Table 1.1). For example, the angle of the hoof wall is steeper for donkey hoof when compared with horse hoof (Fowler 1995). This may cause the hoof wall to undergo different deformation to that of the horse hoof, possibly resulting in the requirement for a different structure to that of horse hoof. The majority of the other observed differences were subjective (Lungwitz and Adams 1913) as no data or quantitative analyses were provided (Reilly 1997). Hifny and Misk (1983) did note, however, that the ratio of lengths of the wall at the toe,

quarters and heels of the forefoot were 3:3:1.5 whereas those for horse hoof horn were reported to be 3:2:1 (Sisson and Grossman 1953). Again, this is likely to indicate a different method of deformation of the hoof capsule between the two species.

Table 1.1 - Summary of Differences Between Donkey and Horse Hoof

Difference	Donkey	Horse
Hoof Size	Smaller than horse hoof (Hifny and Misk 1983)	
Angle of Hoof Wall	Five to ten degrees more upright than horse hoof (Fowler 1995)	
Sole Thickness	Greater thickness than horse hoof (Hifny and Misk 1983; William 1989)	
Number of Laminae	Dermis has fewer laminae than horse hoof (Hifny and Misk 1983)	
Solear Weight Bearing	Debate as to whether sole should bear weight (Reilly 1997)	Sole is *non* weight bearing
Width of Hoof	Proportionately narrower than horse hoof (Lungwitz and Adams 1913)	
Development of Frog	Particularly well developed (Lungwitz and Adams 1913)	
Mechanical Properties	"Tougher" than horse hoof (Lungwitz and Adams 1913)	
Shape of Hoof	Quadrilateral structure with a distinct heel buttress (Fowler 1995)	
Shape of Sole	"U" shaped with a slight flare at the heels (Fowler 1995)	
Ratio of Lengths of Wall at the Toe, Quarters and Heels of the Forefoot	3:3:1.5 (Hifny and Misk 1983)	3:2:1 (Sisson and Grossman 1953)
Perioplic Groove	Widens at heels and fuses with the frog (Hifny and Misk 1983)	Merges with the coronary groove (Sisson and Grossman 1953)
Thickness of Wall at Bearing Border	Maintains a relatively constant thickness around the rim (Reilly 1997)	Tapers radially (Sisson and Grossman 1953)
Tubular Characteristics	Possible differences in tubular characteristics to horse hoof (Hifny and Misk 1983)	
Differences in pattern, distribution and size of tubules	Possible differences to horse hoof (Reilly 1997)	

1.3 Tubular and Intertubular Horn Formation

Fleming (1871b) reported the early identification of tubules existing in horse hoof by Professor Gurlt in Berlin in 1836, although these were then known as horn fibres. Mettam (1896) then referred to tubules as filaments, with the tubular and intertubular material formed from cells covering the papillary and interpapillary areas. This formation of tubules and intertubular horn was confirmed by Nickel (1938 and 1939) who found that the cells of the tips of the papillae formed the medullary or marrow cells at the centre of the tubule. The cells on the walls of the papillae formed the individual tubule cortex and those cells between the papillae formed the intertubular horn. As cornification progresses these cells are moved distally and the medullary cells shrink to form loose debris in the centre of the tubule, finally usually leaving an empty space known as the medulla or marrow (Trautmann and Fiebiger 1957).

Tubules run parallel to the direction of the hoof wall (Balch *et al* 1997). The tubules, surrounded by intertubular horn, can be seen clearly on the external surface of the hoof wall (Bertram and Gosline 1986).

It is believed that tubular and intertubular horn of donkey hoof is formed in the same manner as horse hoof horn.

1.4 Tubule Density

One method of assessing hoof horn quantitatively is to assess the tubule density (TD) of hoof horn. This is found by ascertaining the number of tubules per unit area and is generally expressed per mm^2. A true transverse section for histology is needed with the section taken perpendicular to the line of the tubules. As described earlier in section 1.2.4, tubules are formed from the papillae of the coronary corium. Therefore the papillae are responsible for the tubule density of the *Stratum medium.*

It is believed that this anatomical arrangement of tubules within the hoof wall is responsible for the functional capacity of the hoof (Nickel 1939), together with the

distribution of forces within the capsule (Bertram and Gosline 1987). Variation in tubule density across the hoof wall may act to concentrate stress and lead to controlled elimination of damaged hoof wall to avoid injury to the sensitive structures of the hoof (Newlyn *et al* 1999). Tubule density is also thought to indicate the quality of hoof horn (Dittrich *et al* 1994). The presence of tubules, and thus tubule density, may also be linked with the moisture content within the hoof (*e.g.* Marshall 1945; Baillie and Fiford 1996).

As no information is presently available on the tubule density of donkey hoof horn, the importance of tubule density, assessment of tubule density, and factors influencing tubule density, are discussed for pony and horse hoof horn.

1.4.1 Hoof Function and Mechanical Properties of Hoof Horn

It is believed that tubule density may influence both hoof function and the mechanical properties of the hoof. For example, the orderly proximodistal arrangement of tubular and intertubular horn is believed to provide strength to the hoof wall (Balch *et al* 1997). It is believed that the intimate structural arrangement between tubular and intertubular horn is responsible for the functional capacity of the hoof (Nickel 1939; Schummer *et al* 1981). This arrangement may also be important in stress transfer in allowing locomotory forces to be transmitted to and from the axial skeleton and aiding resistance to forces when the limb is loaded (Nickel 1938; Wilkens 1964). Nickel (1938 and 1939) believed that the tubules acted as vertical struts with the intertubular horn transferring stresses to the tubules. Wilkens (1964) believed that the mechanical strength of the hoof wall was dependent upon tubule density. Tubules may also have a significant effect on the manner in which forces are distributed within the hoof wall (Bertram and Gosline 1986).

Tubule density may be an important parameter in determining hoof properties (Kind 1961; Dietz *et al* 1971 both cited in Geyer 1980). Thomason *et al* (1992) concluded, via studies which identified surface hoof wall strain values, that the microarchitecture of the hoof wall is an adaptation to mechanical function. It was further suggested that intertubular horn contributes more to hoof strength and

stiffness than do the tubules as the direction of peak compressive strains did not correlate strongly with the direction of the tubules. Leach (1980) identified that the hoof wall appeared to be reinforced by the tubules but translated his results of compressive testing to mean that, again, the intertubular horn accounted for a major part of the mechanical behaviour of the hoof wall.

Tubules also act to reinforce the hoof against fracture (Bertram and Gosline 1986; Kasapi and Gosline 1997). This may be because holes in a material tend to produce stress concentrations (Wainwright *et al* 1976). With regard to hoof horn, the medullae would be acting as the holes. Reilly *et al* (1996) believed that tubules contributed to the normal function of the hoof by aiding stress transfer and preventing crack propagation. Further to this they believed that controlled delamination of damaged hoof wall may occur and thus prevent damage to sensitive tissues.

Kasapi and Gosline (1998) carried out a histological study of horse hoof horn and found that the area occupied by medullary cavities in the hoof wall was only 2%. They then estimated that an increase in area of about 2% from including the voids would cause an insignificant increase in flexural stiffness in the x plane. They concluded that the inclusion of voids was unlikely to have been the "singular evolutionary driving force towards the development of hollow hoof wall tubules". Kasapi and Gosline (1998) did, however, consider that the inclusion of tubules into the hoof wall provided a reduction in weight of the hoof but still afforded protection against bending. Following their earlier work, Kasapi and Gosline (1999) then concluded that the purpose of incorporation of the tubules into the hoof wall offered resistance of the hoof to buckling which is similar to hollow fibres being present within a composite.

Tubule density may also be related to the resistance to wear (Schummer *et al* 1981) but no supporting information was provided. This provides another reason for examining the tubule density of donkey hoof horn as Fowler (1995) indicated that donkeys do not generally need to be shod. Conversely, horses are shod to avoid

excess wear to the hoof. A reason for this difference may be that there are, indeed, differences in tubule density between the hoof horn of the two species.

It was also suggested that hoof hardness in cattle and pig hoof may be related to tubule density (Gunther *et al* 1983; Geyer and Tagwerker 1986) although mechanical tests were not carried out. Zoerb and Leach (1978) and Leach (1980) showed a positive relationship between modulus of elasticity and number of tubules. However, their data were not presented as a distribution of tubules per unit area.

On a macro scale, Reilly *et al* (1996) considered the hoof wall to be a composite in terms of its tubular and intertubular structure. A composite can be defined as being composed of stiff, strong fibres, known as the discontinuous phase, in a relatively compliant matrix known as the continuous phase (Vincent 1992). The combined properties of both phases could not be achieved from possessing only one of them in isolation. The tubules are likened to the fibres and the intertubular horn to the matrix. The hoof wall, acting as a laminated composite, would be able to protect the hoof capsule as a whole from catastrophic failure (Reilly *et al* 1996). On a micro scale, keratinous materials are considered to be composites owing to the intermediate filaments (IFs) being present within a matrix (Fraser and MacRae 1980).

1.4.2 Quality of Hoof Horn

As mentioned earlier (section 1.1), many authors refer to the "quality" of hoof horn but this is a very subjective term and should be clarified by objective methods. Rössner (1940) reported Tscherne (1910) as having found that the tubule density of 34-62 for good quality horn was greater than that of 34-57 for poor quality horn. This difference did not, however, appear to be tested statistically. The data were also quoted "per cross section" but the definition of this, together with the units used were not provided. According to Kind (1961 cited in Geyer 1980), tubule density can be used as a parameter for the quality of the horn and corresponds to the high loading to which the horn can be subjected. Dittrich *et al* (1994) believed that an

increase in the number of tubules improved hoof horn integrity. Again, these comments were unsubstantiated.

1.4.3 Moisture Content of Hoof Horn

It has been suggested that tubules may be linked to the moisture content within the hoof (Marshall 1945; Butler 1976 citing Burnisky 1975 and Griffin 1969; Evans *et al* 1990, Vermunt and Greenough 1995; Baillie and Fiford 1996). However, this was contrary to the findings of Zschokke (1885) who found that water uptake does not take place by the horn tubules themselves. Kasapi and Gosline (1998) also concluded that the presence of tubules does not significantly increase hoof wall hydration.

1.4.4 Comparison Between Different Species

The determination of tubule density provides a means of comparing basic anatomical features in hoof horn from different species. As well as macroscopic differences between the hoof capsules of both horses and donkeys, possible differences have been detected at the microscopic level. The early work of Tohara (1948) established that the number of "tubes" was lower in donkey hoof than in horse hoof. Hifny and Misk (1983) also recognised that the hooves of donkeys differ from those of horses in respect of the size and density of horn "tubes". In both cases no data were provided in support of their statements. From a visual assessment of stained hoof sections of both horse and donkey hoof horn in Reilly (1997), there do appear to be differences in tubule density between the two sections. Tubule density can also be used to make anatomical comparisons between equines and other hooved animals (Reilly *et al* 2002c).

1.4.5 Assessment of Tubule Density

Early work mentioning tubule density included that of Tscherne (1910) , cited in Rössner 1940). However, a detailed methodology was not described and, as mentioned in section 1.4.2, the results appeared to be "per cross section". Leach (1980) carried out a tubule count but did not provide specific tubule numbers per

unit area. An outline of methodologies used to measure tubule densities and regions of the *Stratum medium* studied is given in Table 1.2. The part of the *Stratum medium* examined for tubule density was unclear in some cases (Zoerb and Leach 1978; Leach 1980; Pellmann *et al* 1993). The manual technique used by Rössner (1940) was to calibrate the image down the microscope using an ocular micrometer. How this was then translated into a tubule density was not clear. A hard copy of the image is generally generated, either by photographic means (Zoerb and Leach 1978; Reilly *et al* 1996; Reilly 1999) or by a more modern technique of digitisation of images from slides (Pellmann *et al* 1993; Kasapi and Gosline 1997; Reilly *et al* 1998b) although Leach (1980) also used a method of projecting slides onto a wall and then counting tubules.

Previously reported values for tubule densities vary for horse and pony hoof horn and are summarised in Table 1.3.

Following his appeal for quantitative methods for examining hoof horn to be employed (Reilly 1995), Reilly *et al* (1996) established a quantitative and systematic protocol for determining tubule density across the full hoof wall depth (HWD). That is from the *Stratum externum* to the start of the *Stratum internum*. A dorso-palmar decrease in tubule density was found at the midline dead centre of the *Stratum medium*. Results for tubule density prior to this work were not easily compared.

The variation in methodologies and area of hoof wall studied make it difficult to compare the results for tubule density. The method chosen in this project to study tubule density was that established by Reilly *et al* (1996) for pony hoof horn and used by Reilly *et al* (1998) for horse hoof horn. This then enabled a direct comparison to be made between donkey, pony and horse hoof horn.

Table 1.2 - Methods Previously Used to Ascertain Tubule Density in Equine Hoof Horn

Author	Method
Chauveau (1853, in Fleming 1871a)	Unknown
Rössner (1940)	Ocular micrometer and microscope
Zoerb and Leach (1978)	Slides photographed (tubule count)
Leach (1980)	Projection of image from slides (tubule count)
Bucher (1987)	Unknown
Pellmann *et al* (1993)	Semi-automatic image analysis system
Reilly *et al* (1996)	Slides photographed
Kasapi and Gosline (1997)	Digitised images
Reilly *et al* (1998b)	Digitised images
Reilly (1999)	Slides photographed

1.4.6 Zonation of the *Stratum medium*

Table 1.3 indicates that the full hoof wall depth has generally been divided into two, three or four areas for descriptive purposes. These tend to be known as zones. There are two main methods which are used to divide the *Stratum medium* into zones, namely by tubule morphology (Nickel 1938; Nickel 1939; Wilkens 1964; Leach 1980 and Bolliger 1991) or by tubule density. Categorisation into zones has been based on cross sectional and longitudinal studies of tubule shape, size and arrangement of cells of the horn tubules. Bruhnke (1931) and Bucher (1987) based their zones upon the results of differential histological staining. These zones appear to be the same as those established by other workers (Tscherne 1910; Nickel 1938 and 1939; Bolliger 1991). Rössner (1940) identified the outer, middle and inner zones for tubule density on the basis of the histological investigations by Tscherne (1910, cited in Rössner 1940) and were not, however, based on quantitative and statistical methods. The present study concentrates on the zonation of the *Stratum medium* by tubule density.

Table 1.3 - Tubule Density Values for Horse and Pony Hoof Horn

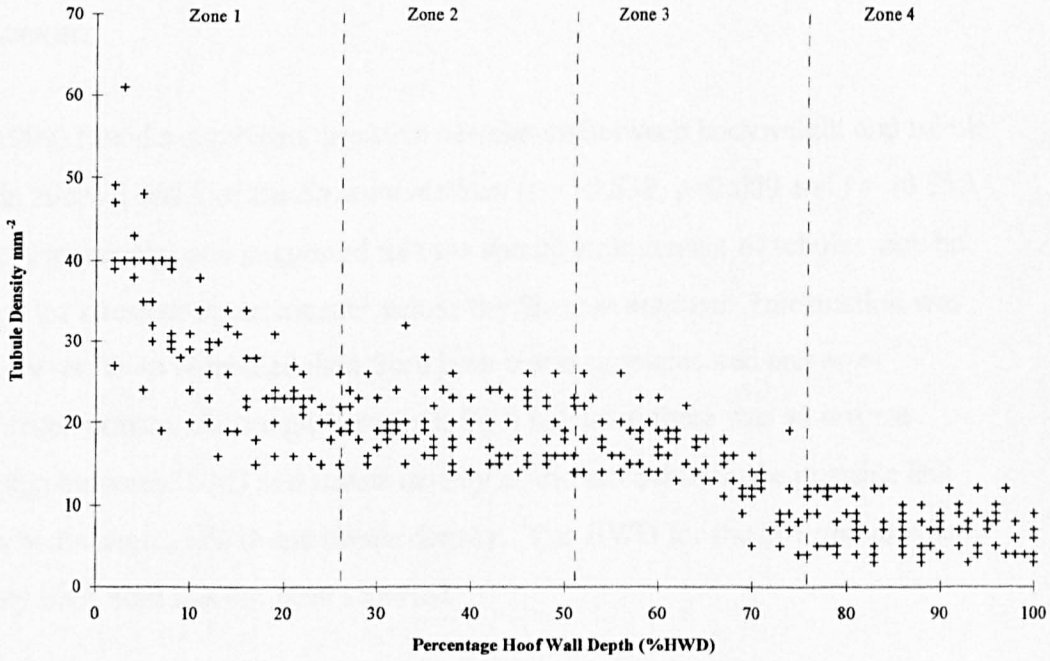
Author	Description of Area	Tubule Density (mm^{-2})
Chauveau (1853) (in Fleming 1871a)	Outer wall Mid wall Inner wall	25-30 15-25 8-12
Rössner (1940)	Outer zone Middle zone Inner zone	15 9 7
Bucher (1987)	Outer zone Inner zone	14 8
Pellmann *et al* (1993)	Unknown	7
Reilly *et al* (1996) and Reilly (1999) (pony)	Zone 1 Zone 2 Zone 3 Zone 4	>27 16-27 8-16 <8
Kasapi and Gosline (1997)	Outer wall Inner wall	25 10
Reilly *et al* (1998b) (horse)	Zone 1 Zone 2 Zone 3 Zone 4	>22 16-22 11-16 <11

The work of Reilly *et al* (1996) and Reilly (1999) showed a dorso-palmar decrease in tubule density at the midline dead centre of pony hoof horn. This indicated a four-zoned, stepped pattern across the *Stratum medium* (Figure 1.4). Each zone equated to approximately 25% of HWD with zone 1 being the outer part of the *Stratum medium* and zone 4 the inner part. Reilly *et al* (1996) and Reilly (1999) argued that this step-like pattern of tubule density may contribute towards the smooth transfer of stress across the hoof wall to the axial skeleton. These authors also believed that the pattern of tubule density within the *Stratum medium* may be important in stopping cracks and resulting in the hoof wall acting as a quadrilaminar ply and shedding an outer zone by the controlled delamination of hoof wall. This may prevent large cracks from developing in the hoof wall and causing damage to sensitive structures.

A further study by Reilly *et al* (1998b) found that the tubule density at the midline dead centre in horse hoof followed a similar four-zoned stepped pattern to that

previously shown for pony hoof. It was concluded by these authors that this four-zoned pattern of tubule density of the *Stratum medium* might be a characteristically equine pattern. However, specific differences in tubule density values and zonal delimitation between horse and pony hoof were observed.

Figure 1.4 - Tubule Density by Percentage Hoof Wall Depth of the *Stratum medium* of Pony Hoof Horn (after Reilly *et al* 1996)



Attention must be paid to these zonations of tubules within the hoof wall as they may provide additional information on hoof function within the capsule (Nickel 1939; Bertram and Gosline 1996; Reilly *et al* 1996; Kasapi and Gosline 1997). The exact reason for this zonation is still unclear. It may be that inter-relationships between tubule density and other features of the hoof such as mechanical properties and moisture content at a zonal level are important as moisture content and mechanical properties may vary across the HWD. These issues will be discussed in detail later in this introduction and also in Chapter 2 and Chapter 6.

1.4.7 Factors Influencing Tubule Density

Factors which are believed to influence tubule density include bodyweight (Reilly 1999), nutrition (Kempson *et al* 1989; Reilly 1999), season (Walz 1951), age (Geyer 1980), breed (Sedlacek 1933, cited in Rössner 1940), genetics (Dietz and Prietz 1981b) and housing (Walz 1951). With the exception of bodyweight, nutrition and breed, these influences have either been reported for cattle or pig hoof horn and not for equine hoof horn.

1.4.7.1 BODYWEIGHT

Reilly (1999) found a significant negative correlation between bodyweight and tubule density in zones 2 and 3 of the *Stratum medium* ($r = -0.839$, $p=0.009$ and $r = -0.750$, $p=0.032$ respectively) and suggested that the spatial arrangement of tubules may be important for stress or strain transfer across the *Stratum medium*. Information was used, however, from combined data from both biotin supplemented and *non*-supplemented ponies. Although Rössner (1940) indicated there was an inverse relationship between HWD and tubule density he did not examine the possible link between bodyweight, HWD and tubule density. The HWD for the *Stratum medium* of donkey hoof horn has not been reported.

1.4.7.2 NUTRITION

Tubule density may be affected by diet as Kempson *et al* (1989) reported an increase in tubule density in pig hoof following dietary biotin supplementation and Dittrich *et al* (1994) reported an increase in the number of tubules following biotin supplementation for horses. In both cases detailed methods for determining these increases, together with statistical analyses, were not provided and therefore these effects may well have been subjective observations. Further work involving supplementation of ponies with dietary biotin revealed a significant difference between tubule density in zone 4 only for the group of four supplemented ponies and the group of four *non*-supplemented ponies (Reilly 1999). Therefore it was

suggested that biotin may have caused an increase in the number of papillae producing the tubules at the corium.

1.4.7.3 AGE

Reilly (1999) did not find a signficant correlation between pony age and tubule density. However, Geyer (1980) suggested there was an inverse relationship between tubule density and age in pigs but this was not examined in his study as he used animals younger than seven months. Dietz and Prietz (1981a) suggested that hoof development in cattle may cease by 2.5 years of age as their results indicated no relationship between the two parameters. It may be that the link with age is as a consequence of an increased bodyweight with age.

1.4.7.4 SEASON

During monthly sampling of cattle hoof, Walz (1951) found that tubule density appeared to decrease during the summer months but no indication was provided as to whether this was a significant decrease. Walz (1951) believed this was due to a weather dependent dehydration of the hoof horn. However, this influence should not have been seen as samples should have been dehydrated during processing. The range appeared small as the results for the whole year actually ranged between 47-60 tubules mm^{-2} (SD 4-10). Distl *et al* (1982) also reported a seasonal change in tubule density for cattle hoof but sampling details were not provided.

1.4.7.5 BREED

Sedlacek (1933, cited in Rössner 1940) believed that tubule density was greater for hooves from "warmblooded" horses than for "cold blooded" horses, although supporting data were not provided.

1.4.7.6 GENETICS

Tubule density was believed to be genetically determined (Walz 1951; Dietz and Prietz 1981b). The heritability of tubule density was 0.32 and 0.38 for cattle

respectively (Walz 1951; Dietz and Prietz 1981b). Distl *et al* (1982) found a much higher heritability of 0.75 for cattle but, again, details were not provided. If tubule density does influence horn quality then heritability may be an important factor not only in livestock production, but also for equines to enable them to avoid foot problems. This would be an area for future investigation.

1.4.7.7 HOUSING

Walz (1951), again for cattle, found there was a significantly lower tubule density for those animals kept loose on slatted floors than those tethered on partially slatted floors although the mean values appeared similar at 53.49 and 55.50 respectively (SD 8.57 and 8.53). Presumably a lack of movement may affect the microstructure of hoof horn owing to a lack of circulation which is normally increased during exercise. Conflicting with the previous results, Distl *et al* (1982) found no significant difference between the tubule density of cattle hoof from animals kept under similar conditions to those studied by Walz (1951). There were, however, no obvious reasons for the conflicting information except that if the data from the study by Walz (1951) is plotted with standard deviation bars, these would overlap, indicating that there was probably no difference.

1.5 Keratinization

The word keratin is derived from the Greek κερασ, meaning "horn" (Fraser *et al* 1972). Keratins are polypeptides and are a family of proteins produced by keratinocytes which die in the terminal stage of keratinization (Fraser and MacRae 1980). Keratinization is the mechanism by which epidermal tissues are rendered tough and insoluble (Mercer 1961). Structures which have undergone keratinization include hair, *Stratum corneum* (of skin), feathers, nails and horns, as well as hooves. Keratinous structures are necessary for protection from the environment, travel, search for food and the maintenance of the species (Lévêque 1994).

The hoof is not solely comprised of keratins but is made up of keratinocytes containing fibrils of keratin within an intracellular matrix. Keratinocytes are joined together by an intercellular cementing substance.

Two types of keratins exist, namely soft keratins such as the *Stratum corneum* of the skin and hard keratins such as hair, nail and hoof. The hard keratin of mammals is based on the α-helix, whereas that of birds and reptiles is based on the β-sheet (Fraser and MacRae 1980).

The majority of research on keratins has been based on hair, wool and skin. Similarities have been shown to exist between hoof and these other epidermal structures (Mercer 1961). The model of the keratinization process is therefore also believed to be similar for hoof horn.

There are various processes believed to be involved in the formation of the hoof wall:

- cornification which is caused by the division of cells within the *Stratum germinativum* (*SG*);

- keratinization which is the synthesis, assembly and interconnection of keratin proteins (Mercer 1961);

- the organisation of keratin proteins into intermediate filaments (Fraser *et al* 1971);

- the synthesis and exocytosis of an intercellular cementing substance and the formation and cross-linking of cell envelope proteins (Grosenbaugh and Hood 1993).

The *SG* is the germinal layer overlying the corium and, for hard hoof horn, is subdivided into the *Stratum basale* (*SB*) and *Stratum spinosum* (*SS*). For soft horn, for example the periople, there is an additional layer present, the *Stratum granulosum*, which exists between the *Stratum spinosum* and *Stratum corneum* (Eckfalck 1990; Bolliger 1991).

The keratinization process starts in the *SB* producing the spiny cells of the *SS*. The *SS* is responsible for the synthesis and assembly of keratin proteins (Bragulla *et al* 1992). A substance containing glycoprotein (Hashimoto *et al* 1992) and phospholipid (Grosenbaugh and Hood 1992) is also formed which has been known as membrane coating granules (Leach 1980), and intercellular cementing substance (Mülling, Bragulla and Budras 1994). This is thought to act as a glue-like substance between cells (Matoltsy and Parakkal 1965, cited in Leach and Oliphant 1983) and may form a permeability barrier (Elias *et al* 1977).

As keratin filaments are synthesised they accumulate and link to each other and also to intermediate filament associated proteins (IFAPs) by disulphide bonds (Pollitt 1992) resulting in a stable "skeletal" structure of fibrils embedded in a matrix. This arrangement may contribute to the structural properties of the cells (Bolliger 1991) and thus, in turn to the structural properties of the whole hoof wall. The fully keratinised cells form the *Stratum corneum*.

The IFs have an α helical symmetry, with the major constituent believed to be low sulphur proteins (Fraser and MacRae 1973). The α helix results from hydrogen bonding between the carboxyl and amino groups of each amino acid. The IFs are embedded in a non-filamentous amorphous matrix containing proteins belonging to a high sulphur containing group or a high glycine tyrosine group (Gillespie 1972). The stabilisation of the matrix is believed to depend on secondary cross linking mechanisms such as hydrogen bonding (Bertram and Gosline 1986) but there are some disulphide bonds within the matrix.

It has long been believed that most of the water sorption in keratins occurs in the matrix and that the intermediate filaments are relatively unaffected by water. This view was based on indirect evidence from mechanical and other properties of keratin, and on more direct evidence based on X-ray diffraction tests (Bendit 1980a).

In conclusion, hoof horn is a highly complex keratinous structure which contributes not only to the mechanical properties of the hoof, but is also likely to influence the moisture content of hoof horn.

1.6 Sampling of Hoof Horn

Hoof horn samples can be obtained from hoof clippings taken at the time of routine hoof maintenance, from morbid capsules or from biopsies. A biopsy of the full hoof wall depth generally involves damaging the sensitive tissues of the foot. This latter technique would involve the use of nerve blocks and painkillers and was not considered in this study on welfare grounds. Partial hoof wall depth samples can also be carried out by biopsy (Ott and Johnson 2001) but these are then only using part of the outer hoof wall.

1.6.1 Clippings

The most readily available hoof samples are clippings which are taken during routine farriery at regular intervals. These intervals can vary but may be, for example, every six weeks dependent upon the amount of growth of the hoof. This farrier attention is needed as hoof growth often exceeds wear and has an effect on foot balance.

However, there are a few factors that should be borne in mind when using hoof samples from clippings. The bearing border is the oldest part of the hoof as the hoof wall grows distally from the coronary border (Pollitt 1992) and may be subjected to damage as it is in continuous use. The bearing border is also subjected to damage by abrasion from the farrier's rasp or from "rolling over" which occurs during the stance phase when the heels have left the ground and the hoof pivots on its dorso-distal margin causing wear at this area. Clippings are also vulnerable to the environment, particularly as that area is in direct contact with the ground (Budras, Schiel and Mülling 1998).

Other authors believe that the mechanical properties of hoof horn at the bearing border may be lower than that at more proximal sites (Bertram 1984; Geyer and Schulze 1994; Hinterhofer *et al* 1998). However, Küng (1991) found no significant difference between the mechanical properties of proximal or distal samples taken from hoof capsules.

The use of clippings does, however, provide a *non*-invasive and continuous supply of full hoof wall depth material in sufficient quantity for the study of hoof horn.

Distal clippings from horse hoof have previously been used successfully for analyses of moisture content (Miyaki *et al* 1974; Küng 1991; Spitzlei 1996; Ley *et al* 1998), mechanical properties (Zenker *et al* 1995; Spitzlei 1996; Ley *et al* 1998), for histological examination (Kempson 1987, 1990) and for trace element analysis (Spitzlei 1996).

The growth of the hoof should also be taken into account. As mentioned in section 1.2.2, the growth rate of donkey hoof horn is yet to be established. Therefore the time taken for full capsule renewal is unknown, although for horses it would be approximately nine months. The clipping material used would be many months old and would have been subjected to environmental insult during this time. The age of clipping material, however, would be more important if, for example, the quarters or heel areas were also to be used for analyses as these would be "younger" owing to there being a shorter distance between the coronary band and the bearing border. Samples taken from clippings are from the midline dead centre sample site only in this project.

It is generally the hoof horn at the distal margin which is under scrutiny in horses as it is the area where shoes are nailed on and is their weight bearing area. It is therefore important that this area can withstand this assault together with that of the environment. As the donkey is generally not shod, the foot interacts directly with the environment. This area should therefore be tested.

Tests on samples taken from clippings of donkey hoof horn are important to establish protocols for work to be carried out on morbid samples at a later stage as morbid donkey hoof horn is not easily available and, as mentioned above, clippings are readily available.

1.6.2 Morbid Samples

Instead of clippings, morbid samples can be used. These provide a full hoof capsule with a much larger sampling area than clippings. The use of morbid samples also overcomes the possible problems associated with the use of clippings by providing a more proximal sampling site thus avoiding any possible damage to the distal part of the wall. Morbid samples have also been used as the duration of a nutritional trial did not allow for full capsule renewal to take place (Reilly 1999).

Although slaughterhouse populations are available for the use of morbid horse hoof horn samples, there is a very limited supply of morbid donkey hoof horn. The history of a slaughterhouse population would also not be known.

1.7 Collection and Storage of Hoof Horn

Samples of horse hoof are known to dehydrate quickly after removal from the animal (Smith 1887; Lambert 1966). The time between sample collection and storage is therefore an important factor when considering the storage of hoof samples. There are no objective measurements reported in the literature which provide details of the dehydration of donkey hoof clippings following removal of samples from the animal. Moisture content was examined in this study in order to establish the values for donkey hoof horn and to assess the influence of moisture content on the mechanical properties of donkey hoof horn. Sample collection must therefore be carried out in such a way as to avoid the loss of moisture.

Moisture content should also be maintained during storage of hoof samples if they are to be used for subsequent moisture content analyses. Storage should also aim to prevent sample degradation. Although sample degradation has not been described, hoof horn samples have undergone degradation when stored in plastic bags at 4°C in a refrigerator (Hopegood, L. personal observations). It is likely that this deterioration was due to the influence of bacteria or fungi.

In many cases reported in the literature there is no mention of storage details for horse hoof although, as shown in Table 1.4, some authors do provide this information. Storage details should be provided as these may subsequently influence the moisture content analyses. For example, Douglas *et al* (1998) stored samples in damp paper towels. If there was a higher moisture content in the paper towels than in the hoof horn then the hoof horn may have absorbed moisture from the paper towels, thus altering the moisture content of the samples.

Moisture content is also known to influence the mechanical properties of hoof horn (*e.g.* Leach 1980; Bertram and Gosline 1987). Any alterations in moisture content would therefore affect the subsequent mechanical testing of samples.

Table 1.4 - Methods Used to Store Hoof Horn

Method	Author
Storage in plastic bags and refrigerated to slow down or prevent sample deterioration by bacterial degradation	Leach (1980), Leach and Zoerb (1983), Bertram (1984), Küng (1991), Hinterhofer *et al* (1998), Ley *et al* (1998), Kasapi and Gosline (1999), Ott and Johnson (2001)
Freezing	-20°C (time unknown) - Bucher (1987), Küng (1991) -10°C for 10 minutes - Kasapi and Gosline (1999) Temperature and time unknown - Ott and Johnson (2001)
Humidified moisture-proof containers at 4°C	Douglas *et al* (1996), Douglas (1998)
Saline solution	Landeau *et al* (1983)
3°C and 41% relative humidity followed by paper towels soaked in 0.9% sodium chloride solution	Wagner *et al* (2001)
65% relative humidity (RH)	Küng (1991), Küng *et al* (1991), Geyer and Schulze (1994), Zenker *et al* (1995), Hinterhofer (1996), Hinterhofer *et al* (1998).
Wrapped in damp paper towels then stored in moisture-proof containers	Douglas *et al* (1998)
Wrapped in Clingfilm at 4°C then in Parafilm[1]	Reilly (1999)

A discussion of some of these methods of storage follows.

1.7.1 Storage of Hoof Horn in Plastic Bags

Plastic bags possess a variety of permeabilities to moisture as different polymers are used. High density polyethylene, which is generally used in their manufacture, has a permeability of 4.8-6.4g/m^2/day/0.025 mm at a temperature of 38°C and a relative humidity of 90%. Plastic bag materials may provide a good moisture vapour barrier

1 Parafilm "M" Laboratory Film, American National CanTM, CT 06836, USA

if the wall thickness is sufficient, at least 0.1 mm, for *e.g.* polyethylene (ASTM D1576 1995). The space around the sample, *i.e.* the headspace, would need to be reduced by evacuation of the bags otherwise moisture may be lost to that airspace. It appeared from the results of Hinterhofer (1996), but was not tested statistically, that samples stored in plastic bags for three weeks showed a much higher stiffness when tested for mechanical properties than those samples stored in plastic bags for twenty four hours. It is assumed that this may be due to either permeability of the bags or loss of moisture to the headspace resulting in loss of moisture from the samples.

1.7.2 Storage of Hoof Horn by Freezing

The effect of freezing on the mechanical properties of hoof samples is disputed. Leach (1980) mechanically tested samples that had previously been frozen to -20°C with those that had not been frozen and showed that freezing lowered the compressive modulus of hoof horn. However, Kasapi and Gosline (1999), who only subjected samples to a temperature of -10°C for a few minutes, showed that freezing had no effect on the stiffness of hoof horn. Sample thawing times prior to mechanical testing for both methods were not indicated. Kasapi and Gosline (1999) also used very small samples which may well have an impact on the results if the same time had been allowed for thawing. Ott and Johnson (2001) froze biopsy samples prior to mechanical testing but did not indicate the temperature used. Again, time allowed for thawing and methods of thawing were not reported. Water loss to the surrounding environment would be expected during this time. Sample storage during thawing would need to take this into account. Further work needs to be carried out in order to assess the effect of freezing on the moisture content of hoof horn to avoid changes in moisture content but is outside the remit of this thesis.

1.7.3 Storage of Hoof Horn in Specific Relative Humidity Environments

Different relative humidity environments are known to alter the moisture content of horse hoof (Bertram 1984; Bertram and Gosline 1987). The relative humidity is the ratio of the vapour pressure of water in the atmosphere compared with the vapour pressure in the air that is saturated with moisture. The relative humidity of the

environment surrounding a sample will influence the moisture content of the sample as water in the form of vapour will be lost to the environment if the surrounding air is drier than the sample. Conversely, if the surrounding environment is saturated, water will be absorbed by the sample.

Many authors have stored and tested samples of hoof horn at 65% relative humidity (Table 1.4) but the reasons for choosing this particular level of relative humidity have not been clarified. Further discussion on the influence of relative humidity environments on hoof horn is provided in section 1.9 of this literature review.

1.7.4 Storage of Hoof Horn in Saline

Landeau *et al* (1983) stored samples in saline prior to mechanical testing but the concentration of the solution was not reported. Following storage of samples at 41% RH, Wagner *et al* (2001) stored samples in paper towels that had been soaked in a 0.9% sodium chloride solution. Presumably these techniques were used to avoid moisture loss from the sample. However, the effect of sample storage in, or adjacent to, a saline solution on moisture content or mechanical properties of hoof horn has not been reported. However, it would be expected that this method would alter the moisture content of the sample owing to movement of water by osmosis.

1.7.5 Storage of Hoof Horn in Clingfilm

The moisture loss of hoof horn samples stored in clingfilm has not been reported. However, Reilly (1999) stored blocks of hoof in clingfilm for two days prior to further sample preparation.

As previously discussed in section 1.7.1, the thickness of plastic bag materials would need to be at least 0.1 mm (ASTM D1576 1995) to provide a good moisture barrier. Clingfilm can be made of polyvinylchloride rather than the high density polyethylene which is used to manufacture plastic bags. Although there are different types of clingfilm, a typical permeability of clingfilm is 155g/m^2/day at 38°C and 80% relative humidity.

Further research would need to be carried out to assess the usefulness of clingfilm to prevent moisture loss from hoof horn samples.

1.7.6 Storage of Hoof Horn in Parafilm

Reilly (1999) wrapped samples of hoof horn in three layers of Parafilm prior to analyses. No data were provided as to whether the use of Parafilm was an efficient method of storage of hoof horn samples without moisture loss.

1.7.7 Time Between Death and Sample Preparation

A variety of approaches has been reported in the literature for preparation and storage of samples of morbid hoof horn. Again, sampling methodologies vary, particularly the time taken between death of the animal and sample preparation, and the time between death and sample testing. Again, these timings are likely to have an effect on the moisture content of the hoof wall unless provision has been made to avoid moisture loss.

Hinterhofer *et al* (1998) prepared samples within two hours of death. Douglas *et al* (1996) and Douglas (1998) obtained feet within twenty four hours of death. Kempson and Campbell (1998) prepared samples betwee 30-60 minutes after death. Wagner *et al* (2001) did not mention the time between euthanasia and sample collection but prepared samples twelve hours after collection and then tested samples within four hours after preparation.

1.7.8 Time Between Death and Sample Testing

Samples have also been taken and tested at varying times following euthanasia. In other instances the time of collection has not been noted but the time to sample testing has been mentioned. Leach (1980) and Leach and Zoerb (1983) carried out tests within 3-4 days of death. Douglas *et al* (1998) tested samples within twelve hours of death and Reilly (1999) tested samples following storage for two days.

Kasapi (1997) and Kasapi and Gosline (1997) used samples within nine days and ninety days of death.

From the variation of methodologies, results and details provided by these different studies no associations could be made between the effect of time taken between death of the animal and sample testing.

1.7.9 Conclusion to Collection and Storage of Hoof Horn

This review of literature emphasises that particular attention should be paid to the collection and storage of hoof horn samples prior to analyses, not only to avoid moisture loss but also to avoid sample degradation.

1.8 Moisture Content of the *Stratum medium*

The functional properties of many biological materials are strongly influenced by the state of hydration as water modulates many of their characteristics (Jackson 1992). Keratin can be classified as a natural fibrous composite comprised of relatively water-impenetrable microfibrils embedded in a water-penetrable matrix (Fraser *et al* 1972). The amount of water within hoof horn may affect its function, quality and mechanical properties. These are discussed further in section 1.8.1 of this thesis.

Even though the role of water in biological processes has been under investigation for over a century (Kuntz and Kauzmann 1974), the review of literature has provided no quantitative information on the moisture content of the *Stratum medium* of donkey hoof horn. Moisture content can be quantified for the full hoof wall depth of horse hoof horn (Table 1.5). The results were reported as percentages of mass and varied considerably from 3.5% to 35.3%. Many moisture content results have not been included in the present section as these have assessed the moisture content of partial hoof wall depths (*e.g.* Leach 1980; Douglas *et al* 1996). These will be discussed later in this chapter.

The assessment of moisture content is normally carried out by a determination of mass loss following dehydration. The methods used to assess horse hoof moisture content in the literature have generally been by different gravimetric methods over differing periods of time. Methods previously used include different temperatures of oven drying and drying at room temperature (RT) (Table 1.5). On detailed inspection there appear to be surprisingly few authors who have used the full HWD to ascertain moisture contents. Examples of authors include Miyaki *et al* (1974) and Spitzlei (1996) (Table 1.5). Comparisons of work have been carried out (*e.g.* the work of Ley *et al* 1998 with Miyaki *et al* 1974) by previous authors even though protocols are often unclear and different methods of dehydration have been used. It is not known whether these different methods produce different moisture content results. There appears to be no standard method of moisture content analysis for hoof horn. The *in vivo*

moisture content is the moisture content that is believed to be in the hoof of the living animal.

Table 1.5 - Equine Hoof Horn Moisture Contents

Author	Number/Type of Animal	Hoof Wall Position	Percentage Moisture Content
Zschokke (1885)	-	Proximal wall Distal wall	28.8 28.5
Smith (1887, cited in Smith 1921)	-	Wall	20.0
Thary (1896), cited in Butler 1976)	-	Wall	16.1
Clement (cited in Caulton Reeks 1905)	-	-	16.1
Smith (1921)	-	Wall	25.0
Gramatzki (1938, cited in Butler 1976)	-	Wall	24.6
Sassen (1938)	13 horses - left fore	-	25.0
Benedetti (1948)	6 horses	Wall	36.3
Miyaki *et al* (1974)	46 horses	Distal clippings	27.1
Butler (1976) Butler and Hintz (1977)	14 x 8 month old Shetland ponies. Front and Hind hooves	Sole border Mid toe Coronary border	27.1 27.8 29.1
Leach (1980)	9 horses 6 horses	Outer wall Inner wall	20.0 27.6
Douglas *et al* (1996), Douglas (1998)	6 horses - 1-15 yr olds. 4 left fores, 2 right fores	Inner wall (n=24) Outer wall (n=24) Medial & lateral quarters	35.5 27.9 32.5
Spitzlei (1996)	Various breeds and ages (n=38)	Distal clippings (3cm)	28.0
Hinterhofer *et al* (1998)	6 warmbloods, 6-13 yr olds	Dorsal and lateral wall	22.0
Ley *et al* (1998)	30 Thoroughbred broodmares, 4-18 yr olds.	Clippings - toe and heel areas	31.2-33.8
Reilly (1999)	4 biotin supplemented ponies 4 *non*-biotin supplemented ponies	Dorsal Wall	3.5-33.9 7.0-29.2

1.8.1 The Importance of Moisture Content in Hoof Horn

The importance of moisture content in hoof horn is discussed on the basis of function, mechanical properties and quality of hoof horn.

1.8.1.1 Function of Hoof Horn

The influence of moisture on the hoof was recognised by Xenophon who noted that "wet and slippery stables ruin even well formed hooves" (Franchini 1998). Bridges (1752) commented that excessive dryness resulted in a brittle hoof. This also caused reduced deformation of the hoof during weight bearing (Lungwitz 1891). Hayes (1903) recognised that moisture had a "softening" and "weakening" influence on hoof horn. Smith (1921), Lambert (1966) and Miyaki *et al* (1974) maintained that proper hoof function was determined almost solely by moisture content. In fact, Smith (1921) went so far as to state that "the entire physiology of the horse's foot is centred around this question of the moisture contained in the horn. The presence of moisture confers elasticity and the absence of moisture creates brittle and rigid horn". Lambert (1966) also showed that excess moisture caused the hoof to lose its ability to hold its size and shape under stress and proposed that the spring-like action of the wall was due to the interaction between the dry outer wall and the moist inner wall. Lambert (1968) also recognised that loss of moisture caused immediate contraction of clippings that were removed from the hoof.

The reason why the function of hoof horn is affected by the presence of moisture is because moisture content is known to influence the mechanical properties of hoof horn.

1.8.1.2 Mechanical Properties of Hoof Horn

As early as 1904, Lungwitz found an increase in tensile elasticity of hoof horn with an increasing moisture content. An inverse relationship has been found between the level of hydration, that is the amount of water present, of hoof horn and its stiffness or its ability to resist deformation (Butler 1976; Butler and Hintz 1977; Leach 1980;

Bertram and Gosline 1986 and1987; Douglas *et al* 1996; Hinterhofer 1996; Hinterhofer *et al* 1996; Kasapi and Gosline 1997; Collins *et al* 1998; Reilly 1999). Miyaki *et al* (1974) reported that the dryness, strength, flexibility and formation of cracks in hoof are controlled by the amount of water present but their study did not support these comments. *In vitro* experiments have shown that an increase in hoof horn hydration is associated with a decreased fracture resistance (Bertram and Gosline 1987; Kasapi and Gosline 1996). Hoof hardness, that is the resistance to indentation, was shown by Naumann (1984) to decrease with increasing moisture content. Küng (1991) showed a negative correlation between tensile strength and sample moisture content.

Conversely, Ley *et al* (1998) found that the percentage moisture content of horse hoof horn was not statistically associated with its tensile strength. However, the mid toe area from all four feet was used for strength testing but the moisture content analyses were conducted on pooled samples from both the toe and heel regions. This therefore encompassed the "younger" horn from the heels. Moisture contents had also previously been shown to vary between front and hind hooves (Zschokke 1885). The dorso-palmar decrease in stiffness across the hoof wall is thought to be related to an increase in water content in the same direction (Leach 1980; Kasapi and Gosline 1997). Hinterhofer *et al* (1998) underlined the necessity to assess the moisture content of samples at the time of testing in order to interpret the mechanical properties of hoof horn.

As water acts as a plasticiser in human *Stratum corneum* (Packer and Sellwood 1978; Van Duzee 1978), this may be the method by which water causes changes to the mechanical properties of hoof horn. A plasticiser is usually an organic liquid of low molecular weight which dissolves in solid polymers which are groups of molecules. The addition of, for example, water, forces the polymer chains apart, making them slide more easily over each other, resulting in greater flexibility of the polymer structure (Ashby and Jones 1994). The larger the number of plasticiser molecules present, the more the attractive forces are disrupted (Van Duzee 1978). This causes biological materials to swell and soften (Vincent 1990). Nissan (1957)

considered the modulus of natural fibres to be related to the number of effective hydrogen bonds per unit volume where a reduction in the water content of the material probably increased the density of effective hydrogen bonds, thereby increasing stiffness. This would cause hoof to act as a rigid polymeric glass, that is that it would be stiff and brittle and would break at low strain.

Biological materials also swell on addition of water as this increases the free space, allowing more freedom of movement for the polymer molecules. Fraser *et al* (1972) noted that, on addition of water to keratin, the microfibrils increased their volume by 11% whereas the matrix increased by 53%. Uptake of water was confined to the matrix and resulted in a greatly reduced mechanical modulus phase in the fully hydrated fibre of wool (Feughelman 1959). This view on the uptake of water by keratin was also based on indirect evidence from mechanical and other properties of keratin, and on more direct evidence based on x-ray diffraction tests (Bendit 1980b). Conversely, it would follow that dehydration affects the properties of the matrix to a far greater degree than the microfibrils, with the mobility of the matrix decreasing, resulting in a high stiffness (Feughelman 1971). Again, these factors may occur in hoof horn.

It can therefore be appreciated that moisture content plays a vital role in the function and mechanical properties of hoof horn although the contribution to these factors is not yet fully understood. As the properties of hoof horn vary with moisture content, it is necessary not only to establish the moisture content at which the measurements are made, but also to study the variation of these mechanical properties with moisture content. The mechanical properties of donkey hoof horn at different levels of hydration were examined in the present study.

1.8.1.3 QUALITY

It has been suggested that hoof moisture content plays a major role in determining the "quality" of hoof horn (Leach 1980, Kempson 1990; Butler 1992; Hertsch *et al* 1996; Budras *et al* 1998). Spitzlei (1996) believed that moisture content was a marker for hoof quality as the study showed that poor quality hoof possessed a

significantly higher moisture content of 30% (SD 4%) compared with 28% (SD 4%) from good quality hoof. Naumann (1984) proposed that if the moisture content of hoof horn was below a certain level it may lead to reduced hoof quality and brittleness but did not provide an indication of the level or any supporting evidence. Budras *et al* (1998) agreed with this and suggested an "optimised" water content is favourable for horn "quality" but the level of moisture content required to achieve this ideal state was not suggested. Lambert (1966) also hypothesised that the governing factor of hoof health was moisture and Marshall (1945) believed that without moisture the horn substance crumbles.

It appears therefore that the moisture content of hoof horn affects the "quality" of hoof horn. However, the term "quality" is very subjective and, again, quantitative parameters are needed to assess what is, indeed, good "quality" hoof horn.

1.8.2 Factors Influencing the Moisture Content of the *Stratum medium*

There are a number of factors that are believed to influence the moisture content of the *Stratum medium* of hoof horn. Ley *et al* (1998) believed that moisture content may be associated with the mineral content of hoof horn but no relationship was found in their study. Other factors that may influence the moisture content of hoof horn include the dermis, tubules, environment, position of sample, disease, age, gender, breed, season and pigment of hoof.

1.8.2.1 DERMIS

Fleming (1871a) believed that the dermal papillae were responsible for supplying moisture to the hoof wall. Zschokke (1885) noted that, despite continuous evaporation of water from the surface of the hoof, the moisture content within the hoof wall itself does not decrease. He therefore assumed that water replacement to the hoof wall occurred via the circulation and decided that the best way of maintaining hoof moisture is by exercise which in turn stimulates blood flow. In later work Smith (1921) ranked the primary sources of hoof moisture as the vasculature of the corium, horn tubule conduction and environmental water. More recently Emery *et al* (1977) suggested that, under dry conditions, the hoof receives

at least 90% of its moisture from the blood and lymph vessels although no proof of this was provided. Butler (1995) believed that a balance of environmental water and systemic water from the blood and lymph supply of the sensitive structures adjacent to the *Stratum medium* was probably maintained through the principle of osmosis, where the water passes from a region of high concentration to a region of low concentration through a partially permeable membrane. Lanovaz *et al* (1998) believed that lack of a vascular supply to the hoof would affect hoof hydration.

The actual contribution of the presence of the dermis to the moisture content is still unknown and would be an area for future research.

1.8.2.2 TUBULES

There appear to be contradictions as to the role of tubules in supplying the hoof with water. Butler (1976) who cited Burnisky (1975) and Griffin (1969) believed that environmental water penetrates into the tubules as a consequence of capillary action. Following this, Butler (1995) stated that horn which is produced from the tips of the papillae is primarily responsible for moisture conduction and regulation in the hoof and that intertubular horn also conducts moisture but to a lesser degree. However, Zschokke (1885) placed hooves and individual pieces of horn into methylene blue and fuchsin solutions in order to assess the penetration of the dyes into the hoof. Detail about the placement of the samples into these solutions and therefore the direction of penetration was not reported. However, the dyes penetrated 0.8 mm into the hoof horn and horn tubules did not take up dye to a greater extent than intertubular horn.

Kempson and Campbell (1998) believed that there were only two possible routes for water to enter or leave the hoof which were either between the cells or through the cells. They then examined absorption of water into hoof sections but did not consider the possibility of the capillary action of tubules. Kempson and Campbell (1998) used a horseradish peroxidase solution as a water soluble tracer. This could then be viewed following reaction with 3'3'-diaminobenzidine tetrahydrochloride. There was minimal penetration of the tracer into the outer 3-5 cell layers of hoof

horn in the intercellular spaces only, whereas the inner layers of the wall showed penetration up to twenty cell layers deep within both the cells and intercellular spaces. The authors suggested that this indicated that the intercellular material in these two regions may be different. Deeper penetration was, however, found for hoof horn containing cracks with the tracer being seen in the intercellular spaces of the intertubular horn only.

Hoof samples from the study of Kempson and Campbell (1998) were placed with the outer hoof wall surface facing down into the tracer but the depth of the tracer was not mentioned. One figure showed the presence of the tracer in some of the tubules. This may have been due to the cut ends of the tubules being exposed to the tracer, by the depth of the tracer within the container covering the ends of the tubules, and then absorption occuring by capillary action. Another figure indicated uptake of the tracer in tubules adjacent to a crack in the sample. It is not clear whether this had traversed the intertubular horn or, again, was absorbed by the cut ends of the tubules. The authors did not comment on this uptake by the tubules.

Kasapi and Gosline (1998) went one step further and looked at the absorption rates of hoof horn by sealing all but one face of blocks of hoof and stored them at 97% relative humidity. This meant that for certain blocks the ends of tubules were directly exposed to a moist environment. There were no significant differences in hydration rates between any of the block faces. Kasapi and Gosline (1998) therefore concluded that tubules do not facilitate the movement of water vapour distally down the hoof.

1.8.2.3 ENVIRONMENT

Another factor affecting the moisture content of hoof horn is environment. There is anecdotal evidence that the moisture content of the hooves of horses kept on shavings or paper bedding is lower than those kept on straw. Ley *et al* (1998) examined hoof samples from three groups of horses. Two groups were kept at grass, one of them was supplemented with concentrated feed and the other had no extra feed. The third group was kept in a barn and given hay. The mean hoof

moisture contents of those animals kept at grass were 31.94% and 31.28% which were significantly lower ($p<0.05$) than the moisture content of 33.83% for barn kept animals. No suggestions were provided for this difference but presumably there was the contribution of urine and faeces to the bedding and this source of water may have increased hoof moisture content.

1.8.2.4 Position of Sample

The moisture content of hoof horn samples within the capsule is known to vary according to the position of the sample within the hoof wall. The presence of a proximo-distal and a dorso-palmar moisture content within the hoof wall is discussed.

1.8.2.4.1 Proximo-distal Moisture Content

There is conflicting information as to whether the moisture content of horse hoof horn is higher in more proximal samples than in distal samples. However, a proximo-distal moisture gradient has not, however, been reported for donkey hoof horn. Butler (1976), Butler and Hintz (1977) and Bertram and Gosline (1987) believed in the existence of this moisture gradient for horse hoof horn. Analysis of the data of Butler (1976) indicated there was only a significant difference between the most proximal sample and the other more distal samples ($p<0.05$, Mann-Whitney U test). There were no significant differences between combinations of the more distal samples. The higher moisture content of the most proximal sample possibly occurred as the section of hoof may have included the papillae which are very close to the blood supply and may, in themselves, have a higher moisture content.

Another reason for the higher moisture content in the proximal sample may have been the presence of the periople. If this does indeed act as a barrier against moisture loss, absence of the periople may then cause the more proximal part of the hoof wall to have a higher moisture content than more distal samples, as shown in the study of Butler (1976).

This moisture gradient may be what Smith (1921) was referring to when he stated that the younger hoof horn nearer the coronary band contained more moisture than more distal samples. Ley *et al* (1998) did not consider this important fact and combined samples from both the toe and heel areas of all four feet when ascertaining moisture content. This therefore means that the moisture contents reported are, in effect, a combination of the two sites which may mask actual differences between the two sites.

Other work contradicts the existence of a proximo-distal moisture gradient. The early work of Zschokke (1885) found similar moisture contents in hoof samples from the proximal wall of 28.8% and those from the distal wall of 28.5%. Leach and Zoerb (1983) also stated that a moisture gradient did not exist in a proximo-distal direction and referred the reader to the thesis of Leach (1980). However, this conclusion did not appear to be outlined in the thesis. Douglas *et al* (1996) also found no significant difference between the moisture content of proximal and distal samples although their distal samples were not taken at the bearing border.

1.8.2.4.2 Dorso-palmar Moisture Gradient

Examples of results from authors indicating a dorso-palmar moisture gradient are presented in Table 1.5. In terms of a dorso-palmar moisture gradient for horse hoof horn, Zschokke (1885), Lambert (1971) and Emery *et al* (1977) noted that the inner part of the wall is damper and has softer horn than the outer wall. However, a dorso-palmar moisture gradient has not been reported for donkey hoof horn.

The majority of studies, until the work of Leach (1980) and Douglas *et al* (1996), concentrated on quantitative values for moisture content for the full hoof wall depth. However, these authors divided the hoof wall into inner and outer wall samples and showed a moisture content of 20% in the outer wall of the hoof whereas that of the inner hoof wall possessed a moisture content of 27.6%. Leach (1980) divided the hoof wall of the right fore of two horses into four sections based on tubule morphology and found a dorso-palmar increase in moisture content.

Douglas *et al* (1996) found the moisture content of outer and inner wall samples from horses to be 27.9% and 35.5% respectively, also confirming a dorso-palmar increase in moisture content.

Thomason *et al* (1992) also believed that the high moisture content of the inner *Stratum medium* resulted in reduced fracture toughness and reduced tensile stiffness. This gradient of stiffness has been interpreted as a mechanism to smooth energy transfer between the wall and the underlying dermis (Leach 1980; Bertram 1984; Bertram and Gosline 1987; Douglas *et al* 1996), although this mechanism has not been defined. Bertram (1984) and Bertram and Gosline (1987) also believed that the effect of hydration provided a mechanism through which the mechanical properties of different areas of the hoof wall can be adjusted to the requirements of the hoof.

Although Wagner *et al* (2001) appeared to consider that moisture content was an important factor in determining the mechanical properties of hooves, they failed to quote moisture contents when determining the moduli of outer and inner wall samples from horse hoof.

It is believed, but has not been shown experimentally, that moisture is lost through evaporation from the external surfaces of the hoof (Leach 1980). This may occur by diffusion of moisture via the intertubular cells throughout the hoof (Smith 1921) but no detail was provided to substantiate this belief. However, Butler (1976) suggested that water may flux from horn cell to horn cell across the hoof wall although further explanation was not provided. Indeed, this moisture gradient may be similar to human *Stratum corneum* in that a concentration gradient arises across the skin and results in a continuous diffusion of water from within the body through the skin and into the environment (Blank *et al* 1984).

More recently Kasapi and Gosline (1997) attributed differences in hoof horn moisture to variation in the protein constituents across the hoof wall and this

variation may be due to different proteins within the regions associating with different numbers of water molecules.

1.8.2.5 Diseased Hoof Horn

It is believed that the moisture content of diseased hoof horn may be different from that of *non*-diseased hoof horn (Goetz 1987; Maclean 1971; Baggott *et al* 1988). It is assumed that any effect of disease which causes an interruption to the blood supply to the hoof would cause an alteration to the moisture content of the hoof horn. Consequently, samples from diseased hoof horn were not examined in this present study.

1.8.2.6 Age

Again, there appears to be confusion as to the influence of age on the moisture content of hoof horn. Naumann (1984) and Spitzlei (1996) showed no significant differences in moisture content with the age of the horse. However, Miyaki *et al* (1974) showed a slightly higher hoof moisture content for the four year old horses (26.1%, SD 4.3) than for those 6 years old and above (27.8%, SD 6.6). As it is not clear as to whether animal age directly influences the moisture content of hoof horn, this was examined as a matter of interest in this thesis.

1.8.2.7 Gender

The moisture content of hoof horn from mares (23.6%, SD 3.5) was lower than that found for geldings (27.3%, SD 4.9) by Miyaki *et al* (1974).

1.8.2.8 Breed

The moisture content of hoof horn from different breeds of horse is not significantly different according to Miyaki *et al* (1974) and Spitzlei (1996).

1.8.2.9 SEASON

Spitzlei (1996) believed that season can affect hoof moisture content but this was not substantiated in her work. Ley *et al* (1998) showed that there may be seasonal variation in hoof moisture content.

1.8.2.10 PIGMENT

From a moisture content point of view no significant differences have been shown between pigmented and *non*-pigmented hoof horn by Benedetti (1948), Miyaki *et al* (1974), Leach (1980), Naumann (1984) and Ley *et al* (1998). However, Hinterhofer *et al* (1998) raised the question as to whether pigmentation does influence the manner or speed of water intake by the hoof horn from its surroundings.

1.8.3 Determination of Moisture Content

In analytical chemistry, if the properties of a sample are influenced by moisture then the water must be removed or be brought to a reproducible level in order to allow a comparison of properties between samples. The level of success in the removal of this moisture depends upon the drying techniques used. Drying may be defined as the removal of volatile substances such as moisture.

The moisture content of a sample is regarded as the ratio of the loss of mass of a test piece when dried under prescribed conditions to its mass at the time of sampling (BSEN 20287 1994). In hoof studies, the majority of authors have used the percentage moisture content by mass but have then not elaborated on how the moisture content has been calculated as there are various methods used to assess the level of moisture within samples.

Moisture contents are normally calculated as a percentage of fresh or original mass (Von Bergen 1963). This method was used by Douglas *et al* (1996) and Reilly (1999) to assess the moisture content of equine hoof horn. Confusion arises as moisture content can also be calculated as a percentage of dry mass and is used to

calculate the moisture content of wool, for example (ASTM D1576-90) and is known as moisture regain. This method was also used by Reilly (1999) to assess hoof moisture regain. These two methods of calculation for moisture content and moisture regain result in differing answers. For example:

Equation 1 - MC as a Percentage of Fresh Mass (FMC_F):

Dry weight (DW)	60 g
Fresh or original weight (FW)	90 g

Method of Von Bergen (1963): $\frac{FW - DW}{FW} \times 100$

$\frac{90 - 60}{90} \times 100 = 33\%$ (moisture content)

Equation 2 - Moisture Regain (FMC_D):

ASTM D1576-90 $\frac{FW - DW}{DW} \times 100$

$\frac{90 - 60}{60} \times 100 = 50\%$ (moisture regain)

The moisture regain results in a much higher percentage as it is on a dry mass basis.

Spitzlei (1996) used dry matter content measured as g/kg. This was calculated as dry mass divided by the original mass x 1000. Küng (1991) used a slightly different method of calculation and used dry mass divided by original mass multiplied by 100. Using the same fresh and dry weights as in the above example this gives:

Spitzlei (1996)

$$\frac{DW}{FW} \times 1000$$

$$\frac{60}{90} \times 1000 = 666 \text{ g/kg (fresh weight)}$$

Küng (1991)

$$\frac{DW}{FW} \times 100$$

$$\frac{60}{90} \times 100 = 66\%$$

Wagner *et al* (2001) used the idea of relative hydration but did not explain the method of calculation. Their moisture content results were 86%. As this figure would be exceedingly high for a moisture content, it is assumed therefore that this result was 86.4% (SD 2.9%) dry matter, therefore resulting in a particularly low moisture content of 13.6% but this was not clear from their methodology.

1.8.3.1 Factors to be Taken into Account When Dehydrating Samples

There are some further fundamental points that need explaining in order to understand the complexities of sample dehydration.

1.8.3.1.1 Temperature, Humidity and Diffusion

As has previously been described in section 1.7, the removal of water from a sample by evaporation is dependent on the vapour pressure difference between the air near the sample and that of the more mobile air above. The sample temperature and that of the surrounding air will also affect the rate of evaporation (Pratt 1986). In general, the amount of water contained in a solid tends to decrease with increasing temperature and decreasing humidity. A wet solid, on exposure to an atmosphere of fixed relative humidity will lose or gain moisture until equilibrium is attained. Moisture leaves the sample surface by diffusion (Willits 1951). This is the movement of molecules from a region of high concentration to a region of low concentration. If the sample is thick, it may require considerable time for this to take place. Further moisture can be removed only by decreasing atmospheric relative

humidity. These important factors should be taken into account when establishing drying regimes.

1.8.3.1.2 Capillarity and Transport of Moisture by Microcracks

The other common mechanisms of moisture penetration into samples are by capillarity and transport by microcracks, each becoming active only after the occurrence of specific damage. Capillarity involves the movement of water molecules along the fibre-matrix interface and occurs only when this interface has been damaged. Transport of moisture by microcracks involves movement and storage of water in microcracks which usually occur from environmental or service conditions. These two mechanisms influence the rate and maximum capacity of moisture absorption (Marom 1985). The movement of moisture into hoof samples via cracks has been described by Kempson and Campbell (1998) and was mentioned in section 1.7.

1.8.3.2 Direct Determination of Moisture Content

Moisture content can be determined either directly or indirectly. Direct determination of moisture content involves the collection of the water evolved from the sample on a chemical absorbent that is specific for water. The increase in mass of the absorbent is then taken as a direct measure of the amount of water present (Skoog and West 1976). This method has not been used previously to assess the moisture content of hoof horn.

1.8.3.3 Indirect Determination of Moisture Content

The main method of indirect determination of moisture content generally involves the loss in mass of a solid during drying. The assumption is made that the loss of mass is equal to the mass of water in the sample (Skoog and West 1976).

1.9 Manipulation of Moisture Content of Hoof Horn

1.9.1 Hydrated Moisture Content

Although the presence of moisture within the hoof is of fundamental importance, it is not always possible to maintain an *in vivo* moisture content during collection, storage and preparation of hoof horn prior to testing. Some authors have used fully hydrated samples for further analyses. These are samples that have been saturated in water until no further mass gain and they are said to be in equilibrium. The use of fully hydrated samples does, however, provide a consistent level of hydration for comparison of all samples. This level of hydration is known in this study as hydrated moisture content (HMC). This method provides a means of representing the amount of water in fully hydrated samples. The moisture content of these samples can, again, be expressed as a percentage moisture content. This then provides a means of being able to compare properties with other samples as the moisture content has, in effect, been normalised.

1.9.2 Assessment of Hydrated Moisture Content

The early work of Fleming (1871a) acknowledged the ability of hoof material to absorb moisture. Slightly later, Zschokke (1885) stated that horse hoof horn existed at 70-90% of its saturation value but the reasoning behind this was not provided.

There is no reported work concerning the moisture content of fully hydrated donkey hoof horn. The early work of Benedetti (1948) established the mass of hoof horn following drying of samples and then placed them in distilled water. No information was provided on the duration of the saturation of samples. The results showed that 0.25 g of water was absorbed per gram of hoof.

The calculation of hydrated moisture content was not really clear until the work of Spitzlei (1996) who determined water absorption by gravimetric means. Samples were taken from the toe area and were dried at 105-110°C and then placed in

distilled water for 14 days until equilibrium mass. The hydrated moisture content was calculated as follows:

$$\text{Hydrated moisture content (g/kg)} = 1000 - \left(\frac{\text{Dry Mass}}{\text{(Hydrated mass}} \times 1000 \right)$$

Samples were taken from good and poor quality hoof. The results showed that good quality hoof had a lower hydrated moisture content of 33% compared to poor quality hoof of 37%. However, the assessment of "quality" of hoof horn was not clear. Reasons for this difference were not provided but this may be due to, for example, the existence of microcracks within the sample which would provide a large surface area for the attachment of water molecules.

Bertram (1984) and Bertram and Gosline (1987) hydrated samples both over distilled water and in distilled water prior to mechanical testing. However, the time taken until equilibrium mass was achieved was not reported. Bertram (1984) found that there was no significant difference between the final moisture contents using these two different techniques, although hydration time was reduced by a factor of two when samples were immersed. The hydrated moisture content was 40.2% which was calculated following drying for at least three days at 80°C although the method of calculation was not clear.

Naumann (1984) determined the hydrated moisture content of horse hoof as 37% by drying samples at 105-110°C for twenty hours and then hydrating them for twelve days in distilled water and reweighing them. The mass differences represented the water absorption ability of the samples and were proportionally expressed, although a description of the calculations was not provided. Following further experiments, Naumann (1984) found that a high moisture content increased the rate of loss of hoof during abrasion tests whilst compressive resistance decreased. He did not, however, see an influence of age, breed, bedding or hoof colour on the water absorption abilities of the hoof.

1.9.2.1 Drying Techniques Used in Assessing Hydrated Moisture Contents

Again, as for determining moisture contents, the drying techniques vary and are discussed in detail in Chapter 2. As with moisture content results, these different drying regimes, together with different times taken for saturation of the hoof may result in different values for hydrated moisture contents and will therefore make comparisons difficult.

1.9.2.2 Zonal Assessment of Hydrated Moisture Content

Kasapi and Gosline (1997) and Kasapi (1997) are the only authors to have calculated hydrated moisture content for different areas across the hoof wall depth. They found that the outer, middle and inner wall possessed a hydrated moisture content of 35%, 41% and 48% which indicated a gradient in a dorso-palmar direction. However, these differences were not analysed statistically.

1.9.3 The Use of Relative Humidity Environments to Manipulate the Moisture Content of Hoof Horn

Controlled humidity chambers have been employed to equilibrate various samples at a particular relative humidity prior to studies on their physical properties (*e.g.* Blank 1952 for skin; Baden 1970 for nail; Mohsenin 1968 for corn; Wildnauer *et al* 1971 and Van Duzee 1978 for human *Stratum corneum*; Alonso *et al* 1996 for rat skin). In this thesis, the use of the term equilibrium describes the situation where the mass remains constant over time. Two of the most common methods of producing environments with different relative humidities are by using different concentrations of aqueous sulphuric acid or different saturated salt solutions (Rockland 1960). Samples are then equilibrated in the environment created by the solutions.

There have been a small number of studies on horse hoof horn where samples have been equilibrated over different saturated salt solutions at different relative humidities (Table 1.6), but in none of the studies were the reasons for this practice explained (Bertram and Gosline 1987; Küng 1991; Geyer and Schulze 1994; Zenker

et al 1995; Hinterhofer 1996; Hinterhofer *et al* 1998). It is assumed therefore that, as hoof horn is greatly influenced by its water content, it is necessary to define the conditions used for physical testing and provide an environment which results in a consistent level of moisture content for each sample. Although Kasapi and Gosline (1997) and Kasapi (1997) conducted tensile tests on fully hydrated samples, they believed that bulk water may accumulate in the medullary cavities of tubules resulting in distortion of the water content measurements. Therefore they dehydrated their samples slightly by equilibrating them in 97% relative humidity environments. Kasapi and Gosline (1998) and Kasapi (1997) also stored hoof samples over a variety of saturated salt solutions, to provide constant relative humidity environments of 33% and 75% prior to testing but moisture contents were not provided. Wagner *et al* (2001) stored whole capsules at 41% RH prior to sample preparation but reasons for the use of this level of relative humidity were not provided.

The importance of testing samples at *in vivo* moisture contents has been appreciated (Douglas *et al* 1996; Hinterhofer 1996; Hinterhofer *et al* 1998). However, it is difficult to maintain this level of moisture content during sample storage and preparation. Use of relative humidity environments may provide a method of rehydrating samples to their *in vivo* moisture content prior to mechanical testing. It is not clear from the authors detailed in Table 1.6 why these particular relative humidities have been used and whether it was expected that an *in vivo* moisture content would be reached by subjecting hoof horn to these particular environments.

Table 1.6 - Relative Humidity Environments for Equilibration of Hoof Horn Samples Prior to Testing

Author	Relative Humidity Environment (%)	Hydrated Moisture Content (%)
Bertram (1984) and Bertram and Gosline (1987)	100 75 53 0	40.2 18.2 11.7 5.5
Küng (1991) and Küng *et al* (1991)	65	13.3
Geyer and Schulze (1994)	65	Not provided
Zenker *et al* (1995)	65	Not provided
Hinterhofer (1996) and Hinterhofer *et al* (1998)	65	15.9
Kasapi and Gosline (1997) and Kasapi (1997)	97 outer wall 97 mid wall 97 inner wall	48 41 35
Kasapi and Gosline (1998) and Kasapi (1997)	33 75	Not provided Not provided
Wagner *et al* (2001)	41	Not provided

1.9.3.1 Different Techniques Used to Ascertain Hydrated Moisture Content

Again, the drying techniques used to assess the hydrated moisture contents of hoof horn vary following equilibration in specific relative humidity environments. These are discussed in detail in Chapter 2.

1.9.4 Sorption/Desorption Isotherms

To investigate the hydration of any substrate Lévêque (1994) believed the first step was to determine the sorption isotherm, which reflects the quantity of water that can bind at a given temperature and relative humidity. A selection of relative humidity environments can also be used to produce a desorption isotherm.

In order to establish the sorption isotherm, samples are dried, weighed and then placed in different relative humidity environments between 0 and 100% and reweighed until there is equilibrium with the surrounding environment. The moisture

content is determined as a percentage of dry mass (Towns 1995). In order to establish a desorption isotherm, samples are fully hydrated and then placed in different environments until in equilibration with the surrounding environment (D'arcy and Watt 1981). Samples are then weighed, dried and reweighed. The moisture content is, again, calculated as a percentage of dry mass. The results are then plotted against relative humidity to produce the sorption and desorption isotherms.

The resulting curves closely reflect the interactions between the chemical groups of a given material and water molecules. The isotherms produced for keratinous materials are generally sigmoidal in shape. These isotherms can be roughly separated into three regions (Hageman 1988). The first region at approximately 0-20% relative humidity consists of binding of water to highly active sites such as charged and highly polar groups and indicates the amount of strongly bound water molecules (Lévêque 1994). An analysis by Watt and Leeder (1968) of the wool-water system established that specific hydrophilic sites such as carboxylic, amino and hydroxyl residues, in addition to peptide groups, are hydrated at low relative humidities. Water uptake at these low levels of relative humidity corresponds to condensation of water molecules in a single layer on a number of particular binding sites (Lévêque 1994). The second region is a transition region from monolayer to multilayer coverage. It occurs with the binding of water to weaker sorption sites such as the peptide backbone and polar surface groups. Additional water binding occurs via clustering at, or near, charged and highly polar groups. The last region occurs with condensation of water at very weak binding sites, layering of loosely held water and through filling of voids created by swelling of the polymer (D'arcy and Watt 1970, Leeder and Watt 1974, Hageman 1988).

The sorption and desorption isotherms generally follow different paths. This is known as hysteresis. The desorption isotherm occurs at a higher moisture content than the corresponding sorption isotherm (D'arcy and Watt 1981). This means that the equilibrium moisture content at a particular relative humidity is higher when a sample is equilibrated by desorption from a higher humidity than if the same sample

had been subjected to sorption. There are many explanations given for the existence of hysteresis. The most common explanation for most proteins is that the penetration of water results in macroscopic polymer deformation and swelling (D'arcy and Watt 1981; Berlin 1981; Kapsalis 1981; Hageman 1988). This swelling of the material may enable new binding sites to be available. During desorption the swollen network may be unable to collapse in a totally reversible manner, thus more binding sites are available for water molecules and hence the desorption isotherm exists at higher values than to the sorption isotherm (Taylor 1952). The amount of hysteresis, known as the relative hysteresis, is calculated as the difference between sorption and desorption values as a percentage of sorption values (Taylor 1952).

Most studies of the effects of water on alpha keratin have used wool and have produced the characteristic sigmoidal sorption and desorption isotherms. King (1945) produced a sorption and desorption isotherm for head horn keratin which also showed hysteresis.

There are no reported results for sorption or desorption isotherms for donkey hoof horn but Bertram and Gosline (1987) studied the fracture properties of horse hoof at different relative humidities and produced a sorption isotherm for horse hoof but this relied on only four levels of relative humidity. They used the results for horse hoof moisture content of 17-24% reported by Leach (1980), and deduced from their isotherm that the *in vivo* moisture content of horse hoof corresponded to a relative humidity of 65-83% with maximum fracture toughness existing at this level. They did not take into account the fact that Leach (1980) used a drying temperature of 60°C which was different to their own drying temperature of 80°C. The 65-83% level of relative humidity to produce an *in vivo* moisture content of 17-24% would therefore be an underestimate. Also, the results from Bertram and Gosline's (1987) study were calculated as a percentage of dry mass. It was not clear how Leach (1980) calculated the moisture content of his samples. The values for moisture content as a percentage of dry mass would be much higher than if they had been calculated as a percentage of *in vivo* mass. The resultant relative humidity necessary

to produce an *in vivo* moisture content would therefore be much higher than the 65-83% suggested by Bertram and Gosline (1987).

Kasapi and Gosline (1997) and Kasapi (1997) used extrapolated data from unpublished work on sorption isotherms to provide estimated moisture contents at 100% RH of 48%, 41% and 35% for samples from the inner, middle and outer hoof wall respectively.

1.9.5 Conclusions from the Literature Review on the Moisture Content of the *Stratum medium*

The literature review has identified that the moisture content and manipulation of the moisture content of donkey hoof horn have not been established. The moisture content of hoof horn is important as it affects the functional and mechanical properties, and possibly the quality, of hoof horn. Many factors may also influence the moisture content of hoof horn. Different methods exist for the calculation of, and determination of, the moisture content of hoof horn.

There is no established data available for the hydrated moisture content of donkey hoof horn. This method provides a consistent level of hydration for comparison of samples if an *in vivo* moisture content has not been maintained.

1.10 Mechanical Properties of Hoof Horn

The mechanical properties of biological and non-biological materials usually depend on the structure and organisation of the material (Wainwright *et al* 1976). Before discussing the mechanical properties of a material such as donkey hoof horn, some standard terminology must be explained.

A load or *force* applied per unit area perpendicular to the line of action of the force is known as a direct *stress*. The deformation per unit length is known as *strain*. *Strength* is the force needed to break something (Gordon 1978).

The mechanical properties of proteins such as keratin are dependent upon how the side chains from the amino acids link with each other and resist the applied forces. The α helix of keratin is stabilised by hydrogen bonds between one peptide link and the third following link (Vincent 1990).

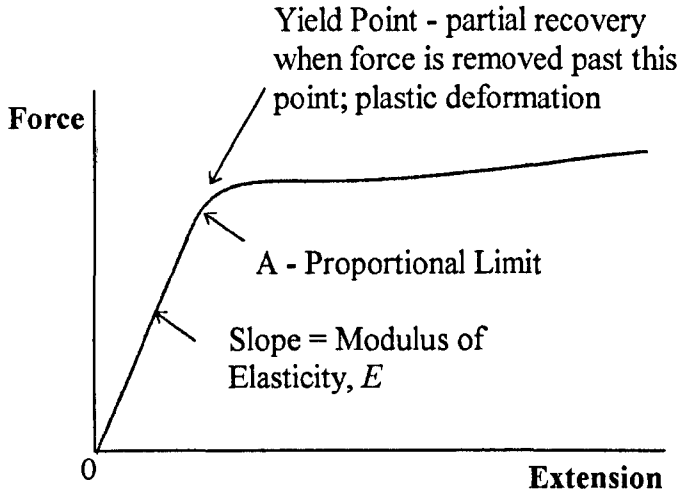
Keratin filaments are cross-linked to associated proteins by disulphide bonds (Bolliger 1991) and this may provide a reason for the strength of keratinous materials (Matoltsy 1976). Providing that the adhesion between the keratinocytes is strong, the length of keratin filaments in relation to their width, may allow for distribution of stresses throughout the material (Fraser and MacRae 1980).

The object of carrying out mechanical testing on hoof wall specimens is to determine the effects of these bonds on the mechanical performance of the material when subjected to loading. It must be remembered that the whole hoof capsule is comprised of complex tissues whose functions depend on the mutual interdependence of its constituent parts.

1.10.1 Assessment of Modulus

The force data collected during testing of a material can initially be expressed by a force-extension graph (Figure 1.5). Extension is the change in dimension of the material. When the initial part of the graph is linear, the material is said to be *Hookean* within this region. The point at which this relationship is no longer linear is the *proportional limit.* Prior to this point the material will return to its original dimensions when the force is removed. The material is said to be linearly *elastic* up to this point.

Figure 1.5 - The Force-Extension Graph



Key:

O-A Elastic region - extension is proportional to force and there is full recovery when the force is removed

Beyond the proportional limit the extension may increase at a greater rate than the rate of increase in force. This is known as the *yield point.* Beyond the yield point the material is described as *plastic* and will not return to its original shape after removal of the load. The yield point is generally agreed to be where the α helical arrangement of the microfibrils breaks down (Fraser *et al* 1972).

In order that the data from one sample of a particular cross-sectional area can be compared directly with another, of a different cross-sectional area, the force is expressed as the stress (Equation 3). This is calculated by dividing the force by the initial cross-sectional area to give the intensity of force on the cross-section or force per unit area (N m^{-2}).

Equation 3

$$\text{Stress} = \frac{\text{Force}}{\text{Area}}$$

Strain (Equation 4) is the extension, that is the change in length of the sample, divided by the initial, or original, length of the sample and is hence a unitless number:

Equation 4

$$\text{Strain} = \frac{\text{Change in length of sample}}{\text{Original length of sample}}$$

The force-extension graph can therefore be used to obtain the stress-strain graph.

The slope of the stress-strain graph, that is the ratio of stress to strain, up to the limit of proportionality is called the modulus of elasticity (*E*) (Equation 5).

Equation 5

$$\text{Modulus, } E = \frac{\text{Stress}}{\text{Strain}}$$

The modulus therefore has units of force per unit area (N m^{-2} or Pa). The magnitude of *E* is a measure of the stiffness or rigidity of the material (Wainwright *et al* 1976).

Sometimes the force-extension graph can show a "toe" region just after testing commences. This is where force is not proportional to the extension. The sample should be pre-loaded with a slight force to minimise the possible effects of movement of the specimen during initial loading. This also ensures that a "toe" region in the force-extension graph is avoided (Jackson 1992).

1.10.2 Mechanical Testing of Hoof Horn

The mechanical properties of hoof horn have, in the past, been determined predominantly by tensile and compressive testing (for example, Zenker *et al* 1995 and Landeau *et al* 1983). The loading on the hoof during *in vivo* conditions involves a complex dynamic loading regime. Excess loading of the hoof capsule *in vivo* may result in catastrophic failure. It is not possible to test the performance of hoof horn according to every different type of *in vivo* condition. It is therefore necessary to define a testing procedure that will produce quantitative results that can then be related to other published work, and to provide a basis on which to compare the effects of other variables such as moisture content and structural differences.

The main approaches to assessing the modulus of horse and pony hoof horn include compression and tensile testing (Table 1.7). Early work, however, favoured cantilever bending, where the hoof samples were fixed rigidly at one end and a load was applied to the opposite end (May 1924; Garnhaft 1925). Other authors have used bending tests to analyse hoof horn (Kasapi and Gosline 1996; Kasapi 1997; Reilly 1999). The moduli results provided from different mechanical tests are presented in Table 1.7 and encompass samples that span the full HWD which were tested at different moisture contents. The mechanical testing of partial HWD samples will be discussed in the section on zonal variation (section 1.11).

Again, as has been identified with moisture content analyses, it is difficult to compare any of these published results as protocols differ. The moduli results from different authors vary from 17 MPa to 14600 MPa. These results depend on the type of test chosen, orientation of sample (BSEN 2746 1998), speed of testing (McElhaney 1966), level of hydration (*e.g.* Butler and Hintz 1977; Douglas *et al* 1996) and sample area (*e.g.* Bertram 1984; Bertram and Gosline 1987).

Table 1.7 - Comparison of Moduli Previously Published for Horse Hoof Horn

	Area of Hoof Tested	Mechanical Test	*E* (MPa)	Crosshead Speed* mm/min^{-1}
***In vivo* Moisture Content**				
Lungwitz (1904)	Unknown	Tensile	10265g**	Unknown
Klein (1931), in May (1925)	Unknown	Tensile	23	Unknown
Goodspeed *et al* (1970)	Clippings	Tensile	17.3-17.6	Unknown
Zoerb and Leach (1978)	Varying samples	Compression	215	0.51
Zenker *et al* (1995)	Coronary horn dorsal wall Proximal Distal Clippings	Tensile (65% RH)	 63 53 46	Unknown
Ley *et al* (1998)	Mid toe clippings	Tensile	21.7-35.32	2
Reilly (1999)	MDC of *SM*	3 point bending	457	2
Hydrated Moisture Content				
May (1924)	Wall	Cantilever bending	500	N/A
Garnhaft (1925)	Pigmented Non pigmented	Cantilever bending	491 471	N/A
Landeau *et al* (1983)	Pigmented areas of *SM*	Compression	216-402	1.33
Bertram (1984), Bertram and Gosline (1986)	MDC of *SM*	Tensile	410-485	5
Bertram (1984), Bertram and Gosline (1987)	MDC of *SM*	Tensile 100% RH 75% 53%	 410 2630 3360	5
Kasapi and Gosline (1996), Kasapi (1997)	Dorsal wall	Dynamic 3 point bending	120-400	N/A
Kasapi and Gosline (1996), Kasapi (1997)	Dorsal wall	Tensile 100% RH	280 320 470 850	4.98 102 1020 3900
Reilly (1999)	MDC of *SM*	3 point bending	345	2
Dried Samples				
Bertram and Gosline 1987	MDC of *SM*	Tensile 0% RH	 14600	 5
Reilly (1999)	MDC of *SM*	3 point bending	1347	2

Key:
SM *Stratum medium*
N/A Not applicable
HWD Hoof wall depth
MDC Midline Dead Centre
RH Relative humidity
MPa Mega Pascals

* See later in this section for definition of crosshead speed

** Results had not been converted to force per unit area

Ideally, when mechanical *in vitro* testing is carried out on hoof horn the test should mimic the conditions of the hoof *in vivo*. Tensile testing, as carried out by many authors (*e.g.* Goodspeed *et al* 1970; Zenker *et al* 1995; Kasapi and Gosline 1996) tests the samples beyond their proportional limit where the material is pulled apart and is therefore not capable of returning to its original shape. Although tensile forces are incurred in the hoof, these do not generally result in catastrophic failure of the capsule. The hoof would also not be solely subjected to tensile forces *in vivo* but would also be subject to compressive forces at the same time. Indeed, Wainwright *et al* (1976) acknowledged that many structures are subjected to both compressive and tensile forces.

Mechanical testing of hoof horn has also encompassed the use of compression testing (Zoerb and Leach 1978; Landeau *et al* 1983). Again, a similar argument arises as for the tensile testing, that is that the hoof, *in vivo*, does not undergo solely compressive forces.

Reilly (1999) carried out 3 point bending on samples of morbid pony hoof horn following a biotin supplementation trial. Three point bending is described later in this section. This method of bending was believed to be supported by the findings of Thomason *et al* (1992) and Douglas *et al* (1996) indicating that the principle forms of deformation that occurred *in vivo* were that of bending and compression. The moduli results for pony hoof horn were 345 MPa, 457 MPa and 1347 MPa respectively for samples that were fully hydrated, at an *in vivo* moisture content and samples that were dried at room temperature (Reilly 1999). Samples at an *in vivo* moisture content were those that had been removed from the animal and had been stored to avoid moisture loss. The effect of moisture content on the mechanical properties of hoof horn has been discussed earlier in section 1.8.1.2. Reilly (1999) used these three levels of moisture content following the work of Kitchener (1987) and Kitchener and Vincent (1987) who carried out 3 point bending tests on Gemsbok head horn at these levels of hydration. The only difference in hydration between the head horn studies and that of Reilly (1999) is that Reilly (1999) dried

samples at room temperature rather than at 110°C as he believed that heat may affect the properties of the keratins.

The effect of drying samples at room temperature is brought into question in Chapter 2 as the effect of drying would depend on the temperature, humidity and air movement within the room and would therefore be unlikely to be reproducible. Although, in the study of Reilly (1999), there was no significant difference between stiffness values for hoof horn from biotin supplemented animals and *non*-supplemented animals at an *in vivo* moisture content, there was a significant difference between stiffness values for both groups when samples were fully hydrated. This indicates the validity of testing samples at different moisture contents as it appears that the difference in stiffness may have been due to the effect of biotin supplementation on hoof horn. A greater range of levels of hydration would provide a more detailed analysis of the effect of moisture content on the mechanical properties of hoof horn.

Further support is provided for the use of bending tests in the examination of hoof horn. Experimental work by Mair (1974), Chang *et al* (1993), Thomason *et al* (1992) and Thomason (1998) and finite element analysis by Newlyn *et al* (1998) have suggested there is biaxial compression at the midline dead centre region of the horse and donkey capsule in the outer wall, together with tension in the inner wall. The early work of Nickel (1938, 1939) reported that the hoof wall was subject to compressive forces only. However, Hood *et al* (1991) evaluated force generation in the hoof wall during loading using transducers capable of discriminating between bending and compressive deformation. They observed that the dorsal hoof wall was subjected to either pure bending, or compression and bending. Pure compression alone within the wall, was not recorded. A detailed protocol and results were not provided in the proceedings. Rooney (1980) also proposed that, during loading, a complex series of bending forces was initiated within the hoof wall. Kasapi and Gosline (1998) believed the presence of hollow tubules may aid the resistance of the hoof against bending forces. The use of bending tests to obtain a modulus derived from the bending stiffness of the beam takes into account both compressive and

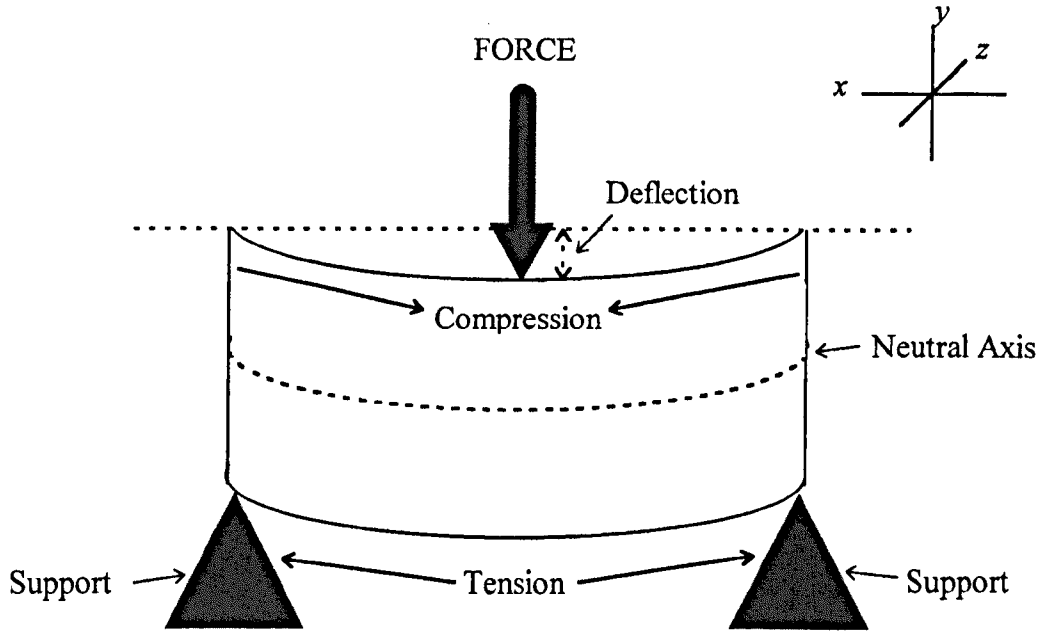
bending forces. A beam can be defined as a structure to which loads are applied at various points along it and is then subjected to bending (Wainwright *et al* 1976).

Tensile and compression tests are also difficult with small specimens due to the difficulty in gripping the tensile test pieces and providing parallel faces for the compression tests. Bending tests are carried out using simply supported specimens which alleviate these problems. The specimen can be tested in the *y* plane with the plane of bending perpendicular to the tubule axes. Bending tests on full HWD clippings from hoof horn cannot be carried out about any other plane owing to the restriction of sample size. It is possible, however, to test samples parallel to the tubule axes if samples are obtained from morbid hooves and thus would be worthy of future investigation.

Bending tests require the measurement of load and deflection of a beam sample. When a beam is subjected to bending, the top surface shortens and is subjected to compression and the bottom surface lengthens and is subjected to tension (Figure 1.6). At the mid-point of thickness of the specimen the material neither extends nor contracts. This is known as the neutral axis (Wainwright *et al* 1976).

The use of bending tests to obtain a characteristic material property is a well established method for engineers and materials scientists. However, the tests are usually used on homogeneous and isotropic, elastic materials. The major characteristic of the hoof wall from a structural point of view is the organisation of tubules aligned along its proximo-distal axis. True composites have a relatively stiff fibrous material embedded in a less stiff component called the matrix. A material is therefore produced which is capable of fulfilling a structural role which combines the high strength and rigidity of the fibre with the energy absorbing capacity of the pliant matrix to produce a relatively strong yet tough material which has good crack resistant properties (Wainwright *et al* 1976).

Figure 1.6 - Beam Subjected to Bending



As mentioned earlier, hoof horn has been likened to a unidirectional composite, with the tubules forming the reinforcement within an intertubular matrix (Kasapi and Gosline 1996; Kasapi 1997; Kasapi and Gosline 1999; Reilly *et al* 1996; Cope *et al* 1998; Newlyn *et al* 1999). Leach (1980), in effect, was also looking at a macro scale composite as he believed that the hoof wall was reinforced by the tubules but that the intertubular material accounted for much of the mechanical behaviour of the hoof wall. Thomason *et al* (1992) lent support to this theory and suggested that the intertubular horn contributes more to hoof strength, stiffness and toughness than the tubules. However, Bertram and Gosline (1986, 1987) and Thomason *et al* (1992) concluded that there was no consistent relationship between the major component of strain and the direction of the tubules and that the hoof acted as a multidirectional composite.

When bending is applied to natural materials such as hoof horn, equations are required to evaluate the flexural modulus (Equation 6). These are derived with certain assumptions:

- The beam is initially straight and unstressed.

- The material of the beam is perfectly homogenous and isotropic.

- The elastic limit is not exceeded.

- The modulus for the material is the same in tension and compression.

- Plane cross-sections remain plane before and after bending.

- Every cross-section of the beam is symmetrical about the plane of bending *i.e.* about an axis perpendicular to the neutral axis.

- There is no resultant force perpendicular to any cross-section.

Two types of static loading are normally used, namely 3 point and 4 point loading. Four point loading is where the beam rests on two supports and is subjected to a symmetrical force at two points. Three point loading is used where the beam rests on two supports and is subjected to a force at the centre point of the beam (Figure 1.6). Cantilever bending has also been used to determine the modulus of elasticity of horse hoof (May 1924; Garnhaft 1925). Cantilever bending is, however, normally recommended for structures in plants such as stems and branches and is easy on long, large specimens. These are held at one end in a clamp and then tested (Vincent 1992). However, the use of this method of testing for samples of hoof horn would be difficult owing to the use of a clamp and the fact that the specimens are not long or very large.

Another variation on 3 point bending has been used by Kasapi and Gosline (1996) and Kasapi (1997) who used a dynamic 3 point bending technique on samples of horse hoof. This is where the sample is subjected to strain which varies sinusoidally with time at a particular frequency. This can produce separate results for the viscous part of a material and for the elastic part of the material (Shadwick 1992). The

frequency ranges used in this case were 0.04 - 200 Hz. Tests were conducted in distilled water resulting in a modulus of 120-400 MPa. Kasapi and Gosline (1999) also carried out dynamic 3 point bending but results were only provided as a ratio of force to displacement. Results from these tests were, again, not directly comparable with previous results for horse hoof.

One factor that needs to be considered in deriving the flexural modulus is whether the deflection measured is due to bending or otherwise. For 4 point bending the shear force is zero over most of the beam. Shear occurs when a force is imposed across a plane of the material, as distinct from a force perpendicular to the plane. Four point bending is not always practical for biological specimens, due to specimen size constraints.

The flexural modulus can be calculated using Equation 6:

Equation 6

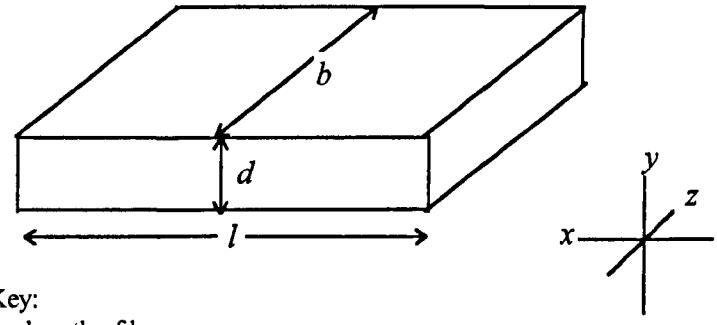
$$E = \frac{W}{\delta_b} \frac{L^3}{bd^3 4}$$

Key:
W = force
δ_b = displacement
L = span
b = sample width
d = sample depth

In 3 point bending the beam is, however, subjected to a shear force rather than simple compression and tension on opposite sides of the beam. The effects of shear are undesirable. An assessment of the error introduced by shear must therefore be calculated to ensure that shear force does not contribute greatly to the beam deflection and hence the modulus. This takes into account the dimensions of the sample and comes into effect particularly when there is a relatively large sample depth compared to length *i.e.* it uses the ratio of the span of the sample to depth of the sample.

The span is the distance between the supports that hold the sample. The breadth of the beam for the main part of this study was the full HWD. The depth of the beam was the hoof wall height (HWH) (Figure 1.7).

Figure 1.7 - Dimensions of Beams



Key:
l = length of beam
d = depth of beam (equivalent to part of hoof wall height)
b = breadth of beam (equivalent to hoof wall depth)

The larger the span to depth ratio, the less the effects of shear but this is difficult to achieve using a biological specimen such as hoof as use of these samples necessitates the use of small specimens. To obtain a span to depth ratio that limits shear, Jackson (1992) and Vincent (1992) had recommended, for other materials, that the span to depth ratio should be greater than ten. The minimum span to depth ratio used in this work was ten.

The calculation of shear deformation uses the shear modulus G. This can be related to the flexural modulus E by the Poisson's ratio (ν). This is the ratio between the longitudinal strain and the lateral strain in either width or thickness of the sample when subjected to mechanical testing. This has not been determined for hoof horn. Chang *et al* (1993) believed that preliminary indications pointed to a Poisson's ratio of 0.4 which Newlyn *et al* (1998) used in their computer model of donkey hoof. Hinterhofer *et al* (1997) used a value of 0.3 in their computer model of horse hoof. Kasapi and Gosline (1997) estimated the Poisson's ratio of 0.45 for mechanical testing of hoof samples which spanned the full HWD. The Poisson's ratio chosen in this study to calculate shear deformation was 0.4 as this was chosen by both Chang *et al* (1993) and Newlyn *et al* (1998).

The effects of shear deformation are determined using expressions for both shear deflection and bending deflection. The shear deflection at the centre of a centrally loaded, simply supported beam of rectangular cross sectional area, can be given by Equation 7.

Equation 7

$$\delta_s \text{ (shear deflection)} = \frac{3WL}{10Gbd}$$

N.B: $E = \frac{2G}{1+\nu}$

Key:
G = Modulus of rigidity (shear modulus)
E = Young's Modulus
ν = Poisson's ratio
b = breadth
d = depth
L = length of beam
W = central load
I = second moment of area

The bending deflection for a centrally loaded beam is:

Equation 8

$$\delta_b \text{ (bending deflection)} = \frac{WL^3}{48EI} = \frac{WL^3 12}{48Ebd^3} = \frac{WL^3}{4Ebd^3}$$

Using a rectangular cross-section: $I = \frac{bd^3}{12}$

For key: see Equation 7.

It is difficult to compare the moduli found from samples of hoof material subjected to tensile or compressive forces with that derived using 3 point bending as the tensile and compressive moduli can be somewhat different and the results are influenced by specimen orientation. Therefore, it should be noted that flexural modulus is an approximate value of the true modulus (BSEN 2746 1998).

Horse hoof horn has also been shown to behave as a linearly elastic material during mechanical testing, displaying a Hookean response in the ranges investigated by Zoerb and Leach (1978), Leach (1980), Landeau *et al* (1983) and Reilly (1999).

There is a lack of information regarding the material characteristics of donkey hoof horn from this type of measurement. Chang *et al* (1993) attached rosette strain gauges to the hoof wall of two donkeys to ascertain the strain pattern exhibited on the hoof wall at the walk and found maximum principal strains occurred at the proximal region of the MDC. Newlyn *et al* (1998) also found similar results when using a computer modelling approach to describe the donkey hoof capsule.

The influence of other factors, such as mechanical anisotropy, strain rates, moisture content, tubule density and pigmentation on the mechanical properties of donkey hoof horn are considered in this section.

1.10.3 Anisotropy of Materials

If a sample differs in moduli according to the direction in which it is tested, it is said to be *non*-homogeneous or *anisotropic*. Given that most other biological materials show anisotropy (Wainwright *et al* 1976), it is perhaps not surprising that the complex structural architecture of hoof horn also shows mechanical anisotropy as was found by Leach (1980) following compression testing of samples. This would enable it to cope with the rapidly changing strains to the hoof capsule brought about by different speeds and directions over different terrain as demonstrated by Thomason *et al* (1992) and Thomason (1998).

Bertram (1984) and Bertram and Gosline (1986) found samples loaded in tension were stiffer when loaded perpendicular to the orientation of the tubules than when loaded parallel to them. Anisotropy has also been indicated in horse hoof where samples encompassed only part of the hoof wall (Leach 1980; Leach and Zoerb 1983; Küng 1991; Douglas *et al* 1996; Hinterhofer *et al* 1998). These partial HWD samples are considered in section 1.11.

1.10.4 Strain Rate

In other biological materials such as bone, the derived material properties can depend on strain rate (McElhaney 1966; Carter and Hayes 1976). Strain rate (Sr) is calculated as strain (ε) per unit time (ε min^{-1}) or as microstrain per second ($\mu\varepsilon$ s^{-1}).

The speed at which the sample is loaded is defined as the crosshead speed. Different crosshead speeds have been used for the derivation of the moduli for horse hoof (Table 1.7) and range from 0.51-3900 mm min^{-1}. If sample dimensions are known, these can be converted to a strain rate. Equation 9 can be used to obtain the maximum strain rate for a rectangular cross sectional sample subjected to bending from the crosshead speed and beam dimensions (BSEN 2746 1998).

Equation 9

$$Sr = \frac{V \times 6d}{L^2}$$

Key:
V = crosshead speed
L = span
Sr = strain rate
d = depth of beam

Butler and Hintz (1977) stated that strain rate can also affect the yield point of horse hoof. Examples of strain rates used during mechanical testing of horse and pony hoof horn are shown in Table 1.8.

Table 1.8 - Strain Rates Used During Mechanical Testing of Horse Hoof Horn

Author	Strain Rate ($\mu\varepsilon$ s^{-1})
Landeau *et al* (1983)	1.73×10^3 - 2.87×10^3
Kasapi and Gosline (1996)	1.6×10^3
Kasapi and Gosline (1996)	32×10^3
Kasapi and Gosline (1996)	33×10^3
Kasapi and Gosline (1996)	70×10^6
Reilly (1999) (pony)	0.7×10^3*

* Calculated from Equation 9

Results from strain gauges attached to the hoof wall of ponies at differing speeds varying from walk to gallop indicated *in vivo* strain rates of 0.02×10^6 to 1.7×10^6 $\mu\varepsilon\ s^{-1}$ (Thomason *et al* 1992). These gauges are only effective on the outer surface of the hoof wall and do not reflect strain changes across the hoof wall depth. Landeau *et al* (1983) carried out compression tests on horse hoof samples using strain rates of between $1.73\text{-}2.87 \times 10^3$ $\mu\varepsilon\ s^{-1}$ and reported no effect on compressive modulus. The reason behind the selection of this rate was not discussed by Landeau *et al* (1983) but was lower than that found by Thomason *et al* (1992) by the use of strain gauges on the hoof wall during walking. Kasapi and Gosline (1996) and Kasapi (1997) believed that the strain rates used in their study encompassed a range of strain rates to which an animal may subject the hoof. These included extremely high strain rates that may be present during crack growth. They concluded that the effect of strain rate on hoof horn was not nearly as great as the effect of hydration of the samples as a five fold increase in strain rate increased the modulus three fold. Until such time as another method of determining physiological levels of strain rate across the HWD is designed, these levels at least provide a means of comparing studies.

1.10.5 The Influence of Moisture Content on the Mechanical Properties of Hoof Horn

The influence of moisture content on the mechanical properties of hoof horn has been discussed previously in section 1.8.1.2. However, a few further comments are needed as the influence of full hydration on the mechanical properties of hoof horn was not discussed.

The results of Bertram (1984) and Bertram and Gosline (1987) for fracture tests for fully hydrated samples were lower than those at an *in vivo* moisture content. They therefore concluded that hoof wall functions under hydration conditions that maximise fracture resistance. This, again, reinforces the use of different levels of hydration for comparative purposes. Indeed, it may be that other mechanical properties of hoof horn appear to be maximised at an *in vivo* level of hydration.

Further investigations of the mechanical properties of horse hoof at full hydration have also been carried out (Kasapi and Gosline 1996; Kasapi and Gosline 1997; Kasapi 1997). In these particular cases it was not clear why mechanical testing was carried out at full hydration, even though Kasapi and Gosline (1996) acknowledged that full hydration was an unusual condition for the hoof wall.

Many authors do not report the moisture content of samples used for mechanical testing following hydration of samples at different levels of moisture content (Geyer and Schulze 1994; Zenker *et al* 1995; Kasapi 1997; Kasapi and Gosline 1998). Therefore it is difficult to compare mechanical testing results between studies as, although the final results may be similar, the moisture contents of the samples could be very different. In order to compare mechanical properties, steps should be taken to ensure the moisture contents of the samples are similar.

1.10.6 The Effect of the Tubular and Intertubular Structure on the Mechanical Properties of Hoof Horn

The role of tubules within the hoof wall is still considered to be a mechanical one. However, tubule functions are very diverse and difficult to quantify (Kasapi and Gosline 1997). The effect of tubule density on the mechanical properties of hoof horn has been discussed in section 1.4.1. The additional influence of the tubular and intertubular structure on the mechanical properties of hoof horn is discussed.

Thomason *et al* (1992) reported no relationship between the direction of the principal strain and tubular or intertubular horn configurations, although Chang *et al* (1993) remarked that the strain pattern was aligned with the major functional axes of the hoof wall in horses and donkeys. Again, both these groups used strain gauges attached to the outer hoof wall and the results therefore do not encompass the full HWD.

Using fracture tests, Bertram and Gosline (1986) showed that the intertubular horn provided an "impressive resistance to fracture in any direction". This was confirmed

by Kasapi (1997) and Kasapi and Gosline (1997) who found that the tubules retarded crack propagation by diverting crack growth.

Composite theory predicts that the stiffness of a fibre-reinforced composite will depend upon the orientation of the fibres relative to an applied stress and upon the mechanical properties and volume fractions of the fibre and matrix phases (Wainwright *et al* 1976). Knowledge of this detail may be useful in understanding the mechanical behaviour of hoof horn but is outside the scope of this thesis.

When studying the microarchitecture of tubules, Nickel (1938b) characterised tubules into two types, those with predominantly steeply angled spirals and those with predominantly low angled spirals. This refers to the IF alignment within the tubule cortex. Kasapi (1997) and Kasapi and Gosline (1997) carried out a more comprehensive study of the IF organisation in cells of the tubule cortex and the intertubular horn and related this to their mechanical findings. Their results showed a change in alignment of the IFs within a single tubule cortex and also differences according to tubule position within the hoof wall. IF alignment within the intertubular horn of the inner wall was perpendicular to the tubule axis. This gradually changed across the hoof wall depth and was almost aligned with the tubule axis in the outer wall.

Kasapi (1997) and Kasapi and Gosline (1999) believed that the volume fraction of the fibres, which is equivalent to the percentage IF content, is also important as their results suggested that IF volume fraction influenced the mechanical properties of hoof horn. They found that the IF volume fraction for tubular and intertubular horn from the inner wall was 23%. IF volume fractions for intertubular horn in the middle and outer regions were 31% and 30% respectively. However, these authors could not provide statistical confirmation of the effects of IF volume fraction on the mechanical properties of hoof horn. The volume fraction of fibres in wool was 62% (Fraser *et al* 1972) and for sheep and cattle horn was 65% and 61% respectively (Feughelman 1979). These results were higher than those found by Kasapi (1997) and Kasapi and Gosline (1998) for horse hoof horn. Examination of the IF

alignment for donkey hoof has yet to be carried out. Testing of the full hoof wall depth would encompass the possible effects of both IF alignment and IF volume fraction. The IF alignment of donkey hoof horn would be another important research area for the future.

1.10.7 The Influence of Pigmentation on the Mechanical Properties of Hoof Horn

Pigmentation of the hoof has traditionally been implicated as an important factor in the durability and strength of horse hooves (Dollar 1898; Wiseman 1973). However, Garnhaft (1925) found the modulus of wall horn to be 491 MPa for pigmented hoof compared to 471 MPa for *non*-pigmented hoof. Klein (1931) did not find a difference in tensile strength between pigmented and *non*-pigmented horn, with them both having a median value of 23 MPa but this was not analysed statistically.

Bertram (1984) found there was no significant difference in fracture properties between differently pigmented hooves at 100% RH, but there was a difference at 53% RH. This was explained by low sample numbers and differences between individuals. Ley *et al* (1998) found no significant difference in tensile strength due to hoof colour. It is likely therefore that pigmentation of hoof does not affect the mechanical properties of hoof horn.

1.11 Zonal Mechanical Testing

Reilly *et al* (1996, 1998b) believed that it was possible that differences in zonal tubule density would confer differences in mechanical properties. Reilly *et al* (1996, 1998b) believed that the transitions between the four zones which were ascertained by studying the tubule density for pony and horse hoof, may allow for controlled delamination of the wall by acting as a quadrilaminar ply. The wall could therefore be divided into four layers with the outer layer being lost if subjected to damage, leaving the remaining three layers intact. The transition region between two zones of differing morphology and physical properties may result in decreased cohesive abilities between the zones (Bolliger 1991).

The mechanical properties of the different regions of the hoof wall depth from donkey hoof horn, together with the influence of moisture content on these properties, have not been investigated.

As the structural organisation of the hoof wall is believed to have a functional significance (Bertram and Gosline 1986), different parts of the hoof wall may require a range of abilities, possibly resulting in distinct mechanical properties for differing areas. Reilly *et al* (1996 and 1998b) divided the HWD of pony and horse hoof into four zones based on tubule density. It may be that these zones also exist for donkey hoof horn as Reilly *et al* (1996 and 1998b) believed that this zonal arrangement may constitute an equid pattern. As indicated in section 1.4.1, tubule density may influence the mechanical properties of hoof horn and, in turn, may influence the zonal mechanical properties of hoof horn.

For horse hoof, some authors have not used the full HWD for their mechanical testing, but have removed part of the outer wall and part of the inner wall (Butler 1976; Butler and Hintz 1977; Hinterhofer 1996; Hinterhofer *et al* 1998). Leu (1987) and Geyer and Schulze (1994) used samples from the outer wall for tensile testing. Zenker *et al* (1995) and Ley *et al* (1998) used clippings for tensile testing and although the exact

region of hoof tested was not provided, it appeared from the diagrams in both papers that the mid part of the HWD was being tested.

Other workers have divided the hoof wall into two sections for mechanical testing, namely an inner wall section and an outer wall section. These have shown a lower modulus for the inner wall samples than for those from the outer wall, indicating a gradient of stiffness across the HWD (Leach 1980; Leach and Zoerb 1983; Küng 1991; Zenker *et al* 1995; Douglas *et al* 1996; Douglas 1998; Wagner *et al* 2001). Kasapi and Gosline (1997) also found a gradient of stiffness and believed that particular mechanical properties are required in specific regions across the HWD. The outer wall must resist cracks and minimise abrasion, but the inner wall must be capable of transferring loads to the dermis. Kasapi and Gosline (1997) also believed that a stiffness gradient exists across the HWD as high stresses would result between an otherwise stiff outer and a soft inner wall. Kasapi and Gosline (1999) believed that the gradual change in stiffness across the wall, partly arising as a result of the proximity of the tissue to a source of moisture (Leach 1980) provided for a more gentle transfer of loads to the dermis. It is likely that the significant differences in the modulus between the inner and outer wall samples from Douglas *et al* (1996) may be explained as a result of sample moisture content differences.

Further mechanical tests have been carried out with the hoof wall divided into three sections (Küng *et al* 1993; Kasapi 1997; Kasapi and Gosline 1997). Reasons for dividing the wall into two or three sections were not provided. Kasapi (1997) and Kasapi and Gosline (1999) have progressed even further by testing individual tubules and intertubular horn from different areas of the hoof wall by tensile testing. The results from the mechanical tests carried out by other authors for partial hoof wall depth samples are shown in Table 1.9.

As would be expected, and as for full HWD samples, there was an inverse relationship between mechanical properties and moisture content for zonal areas of the HWD (Leach 1980; Bertram and Gosline 1987; Küng 1991; Douglas *et al* 1996; Hinterhofer 1996; Hinterhofer *et al* 1998).

Table 1.9 - Comparison of Moduli Previously Published for Different Hoof Wall Areas

	Area of Hoof Tested	Mechanical Test	*E* (MPa)	Crosshead Speed mm min^{-1}
(a) *In vivo* Moisture Content				
Leach (1980)	Outer wall Inner wall	Compression	420-2224 261-938	1.27
Leach and Zoerb (1983)	Inner wall Outer wall	Compression	237 355	1.3
Leu (1987)	Dorsal and lateral outer wall	Tensile 65% RH	59	N/A
Küng (1991)	Dorsal wall Proximal Outer zone Inner zone Dorsal wall Distal Outer zone Inner zone	Tensile 65% RH	 61 42 61 42	N/A
Küng *et al* (1993)	Outer zone Middle zone Inner zone	Tensile 65% RH	60 70 40	N/A
Geyer and Schulze (1994)	Dorsal and lateral outer wall 3cm from coronary border	Tensile 65% RH	58-70	N/A
Zenker *et al* (1995)	Coronary horn dorsal wall Proximal Distal Proximal Inner Proximal Outer Clippings	Tensile (65% RH)	 63 53 41 63 46	N/A
Douglas *et al* (1996) Douglas (1998)	Dorsal outer wall Dorsal inner wall Dorsal outer wall Dorsal inner wall	Tension Tension Compression Compression	955 502 1004 523	5 5 5 5
Hinterhofer (1996)	Dorsal wall	Tensile *In vivo* MC 65% RH 3 point Bend	 543-2171 1774 599-2558	 1 1 5
Hinterhofer *et al* (1998)	Dorsal wall Dorsal and lateral wall	Fresh 65% RH Tensile/ bending	762 1802	1
Ley *et al* (1998)	Mid toe clippings	Tensile	22-35	2
Wagner *et al* (2001)	Outer *SM* *SMZA**	3 point bending	182 98	5.1

	Area of Hoof Tested	Mechanical Test	*E* (MPa)	Crosshead Speed mm min^{-1}
(b) Hydrated Moisture Content				
May (1924)	Dorsal outer wall Dorsal inner wall	Cantilever bending	412 383	N/A
Butler (1976)	"mid toe region"	Compression	3-5	1.27
Butler and Hintz (1977)	"mid toe region"	Compression	4	1.27
Kasapi (1997), Kasapi and Gosline (1997)	Dorsal inner wall Dorsal middle wall Dorsal outer wall	Tensile	300 430 560	1.02
Kasapi (1997), Kasapi and Gosline (1999)	Inner wall tubules Inner wall intertubular horn Middle wall tubule Middle wall intertubular horn	Tensile 100% RH	470 80 290 140	2
(c) Dried Samples				
Hinterhofer (1996)	Dorsal wall not full HWD	Tensile	3346	1
Hinterhofer *et al* (1998)	Dorsal and lateral wall not full HWD	Tensile/ bending	1636-8650	1

Key:

HWD	Hoof wall depth
RH	Relative humidity
SM	*Stratum medium*
SMZA	*Stratum medium zona alba*
MPa	Mega Pascals
N/A	Not applicable
*	See text for description

As this study concentrated on using 3 point bending, the majority of the following discussion concentrates on the work from authors that have used a similar mechanical testing technique.

Hinterhofer (1997), Hinterhofer *et al* (1998) and Wagner *et al* (2001) have carried out 3 point bending tests on partial HWD of horse hoof horn. However, Hinterhofer (1996) and Hinterhofer *et al* (1998) did not provide reasons for testing samples by this testing method. Wagner *et al* (2001) believed, following the work of Hood *et al* (1991) that testing under bending conditions resembled the natural physiologic motion.

Again, as with the analyses of moisture content, the results in the literature are difficult to compare as the type of test, hydration conditions, sampling methods and orientation of samples for testing vary.

Hinterhofer (1996) did not provide full details of sample preparation. It was assumed that the samples tested perpendicular to the line of the tubules were prepared in the same way as those in Hinterhofer *et al* (1998). Samples were initially cut with dimensions of 8 mm x 8 mm x 50 mm from the MDC, without using the most proximal or the most distal 1.5 cm of the hoof wall. Samples were then reduced in size to 3-5 mm x 3-5 mm x 40-50 mm by removing a small amount of external hoof wall and the remainder from the internal part of the wall. However, there was no description of the samples prepared to be tested in the *y* direction. This is confusing as the sample length was 40-50 mm which would be very difficult to achieve with the curved shape of the hoof wall. No mention was made of using curved samples. If this was not the case then sample testing may not have taken this fact into account and may have covered possibly all zones of the hoof wall. The actual hoof wall height used for removal of the sample was also not indicated. If the sample depth was 5 mm then, with a span of 30 mm, this would produce a low span to depth ratio of six, and would result in an underestimate of the moduli by 5% due to the results of shear. The results from Hinterhofer (1996) are shown in Table 1.10.

Samples were tested from Horses 1-6 and Horse 7 in the *y* direction, that is the same direction as in the present study, but following storage for three weeks and twenty four hours at 2°C respectively.

Table 1.10 - Results from Hinterhofer (1996) for 3 Point Bending Tests on Horse Hoof

Horses	Sample Preparation	*E* - Perpendicular to Tubule Axis (MPa) (Standard Deviation)	*E* - *y* Plane (MPa) (Standard Deviation)	Moisture Content (%)
1-6	3 weeks at 2°C	2558 (137)	2349 (212)	-
7	24 hours at 2°C	864 (426)	1106 (163)	18.9
8	24 hours at 2°C 3 days at 2°C	600 (141) 934 (283)	- -	15.8 15.8

At first inspection the samples of Hinterhofer (1996) did appear to show anisotropy. However, the standard deviations did show overlap with each other. It is unlikely therefore that the samples were anisotropic. As the confusion exists over sample preparation and methodology, this would need to be confirmed to ensure that samples did not in fact show anisotropy.

Hinterhofer *et al* (1998) used a test speed of 1 mm min^{-1} which was slower than the 5 mm min^{-1} used by Hinterhofer (1996). It is not clear from the information provided in Hinterhofer *et al* (1998) in which direction the samples were tested, that is from lateral to medial, medial to lateral, in a dorso-palmar direction or palmar dorsal direction.

Wagner *et al* (2001) carried out 3 point bending tests on the outer *SM* and *SM zona alba* (*SMZA*) of horse hoof horn as they believed the *SMZA* may serve as a buffer zone between the rigid hoof wall and pedal bone and the laminar tissues. The *SMZA* does not exist in donkey hoof horn as the full HWD in *non*-pigmented hooves is all one colour (Hopegood, L. personal observations). Wagner *et al* (2001) tested longitudinal sections from the MDC and discarded the proximal and distal 1 cm sections. Samples were tested in a dorso-palmar direction and then in a palmar dorsal direction. The results are outlined in Table 1.11. There were significant differences between the modului for *SM* and *SMZA* samples tested in both directions ($p<0.01$). The results for

the *SMZA* tested in a dorso-palmar direction were not, however, provided. It is likely that the differences shown above were, however, due to the moisture content of the sample and possibly the different strain rates. Although these workers had mentioned the importance of moisture content, this fact had not been taken into account in their experiment.

Table 1.11 - Moduli for the *Stratum medium* and *Stratum medium zona alba* (Wagner *et al* 2001)

Direction of Testing	*Stratum medium* (MPa)		*Stratum medium zona alba* (MPa)	
	Range	Mean (SD)	Range	Mean (SD)
Dorso-palmar	89.2-232.6	181.5 (41.8)	Not provided	Not provided
Palmar dorsal	97.7-240.9	187.6 (41.3)	60-163.1	98.2 (36.8)

Samples were also tested for yield strength by continuing the test past the proportional limit. Samples tested that are taken past the proportional limit where stress is no longer proportional to strain undergo plastic deformation and only recover partially. Samples were then tested in the opposite direction. Therefore it was difficult to understand the reasoning behind retesting of samples in the opposite direction if samples were unable to recover fully.

Sample dimensions were not provided by Wagner *et al* (2001). However, a span of 25.4 mm was used. The span to depth ratio should be considered. If, for example, the HWD was 10 mm and the *SMZA* was 4 mm, the span to depth ratio of the outer *SM* samples would have been 4.2, whereas that of the *SMZA* would have been 6.4, both of which are below the recommended ratio of ten. Therefore there would have been an underestimate of the moduli by 10% for the outer *SM* due to the effects of shear but this was reduced to 5% for the *SMZA*.

The strain rates used by Wagner *et al* (2001) were also very high. Using the sample dimensions indicated above, together with a speed of testing of 5.1 mm min^{-1}, the strain rate for outer *SM* samples was 0.12×10^6 $\mu\varepsilon$ s^{-1} and for the *SMZA* was 80×10^3 $\mu\varepsilon$ s^{-1}. The authors did not take into account the different strain rates that would be brought about by the differing sample depths. This may have made a slight difference to the moduli between the two different areas.

Despite the fact that Wagner *et al* (2001) stated that hydration is a biological factor of paramount importance on the material properties of hoof horn, their result for relative hydration of samples was not split between the *SM* and *SMZA* and was stated to be 86.4%. This one figure was assumed to be the mean of all the samples.

During preparation of samples Wagner *et al* (2001) stored them at 3°C and 41% RH, which would have had a dehydrating effect on hoof horn. Following preparation, samples were wrapped in towelettes moistened with physiologic saline (0.9% NaCl). As mentioned earlier, the effect of this storage method on the moisture content of hoof horn is not known.

1.11.1 Anisotropy of Partial Hoof Wall Depth Samples

Following on from the discussion in section 1.10.3, the effect of sample orientation on the mechanical properties of hoof horn, and thus the anisotropy of samples, for partial HWD will be discussed.

The question of anisotropy still exists for partial HWD samples. Leach (1980) and Leach and Zoerb (1983) found a higher compressive stiffness in dorsal outer wall samples when loaded perpendicular to the tubules than parallel to them. These authors believed that three factors contributed to this anisotropy, namely the arrangement of the tubular and intertubular horn, water content and pigmentation. They believed that for vertically loaded outer wall specimens there was a reduction in the amount of tissue available for supporting and resisting a load owing to the presence of the tubule medullae. However, in laterally loaded specimens the tubules were not acting as voids but because of their elliptical shape may have reduced the effect of laterally directed forces. The thick cortex was believed to act as a buttress to the tubule during horizontal compression.

Küng (1991) tested samples from the middle of the *Stratum medium* and found that the tensile strength of samples tested longitudinally with the tubule axis was significantly lower ($p<0.01$) than those tested mediolaterally with the tubule axis.

Douglas *et al* (1996) investigated the mechanical anisotropy of horse hoof and found a significant difference between dorsal outer wall samples tested parallel to the tubules and those tested perpendicular to the tubules for tensile testing. The stiffness of samples loaded in tension parallel to the tubules being greater than those loaded perpendicular to the tubules. Bertram and Gosline (1986) found that samples loaded in tension were stiffer when loaded perpendicular to the orientation of the tubules than when tested parallel to the tubules.

Wagner *et al* (2001) believed that because of the unidirectional tubular configuration, the *SM* is an anisotropic tissue. However, their results did not show this as there were no significant differences between moduli when samples from horse hoof horn were tested in two directions by 3 point bending.

As mentioned earlier, the anisotropy of donkey hoof horn samples is unknown. Morbid samples would be needed to assess this property for full HWD samples. This could, however, be achieved for smaller samples from zones of the full HWD and would be worthy of further investigation.

1.11.2 Conclusions from the Literature Review on the Mechanical Properties of Hoof Horn

It is evident from the literature review that no information was available on the mechanical properties of donkey hoof horn and the interaction of these properties with moisture content. A mechanical testing procedure needed to be established that resulted in quantitative data for the mechanical properties of donkey hoof horn that can then be related to previous studies on pony and horse hoof horn. A range of levels of hydration would provide a more detailed analysis of the effect of the moisture content on the mechanical properties of donkey hoof horn. The use of 3 point bending to examine the mechanical properties of donkey hoof horn comprises a combination of compressive and tensile testing.

1.12 Overall Conclusions from the Literature Review

From the literature review it can be seen that there is little quantitative information about the *Stratum medium* of donkey hoof horn. Gross anatomical differences between donkey and horse hoof horn have been identified. It is not clear whether further differences in, for example, tubule density, moisture content and mechanical properties of hoof horn exist between horse and donkey hoof horn. Studies that have been carried out on horse hoof horn have also been difficult to compare owing to differences in methodologies, including sample collection, preparation and storage. Many studies do not provide statistical analyses of data. The lack of standardised methods for the analyses of hoof horn must be addressed in order to progress in this particular field of study.

1.13 Aims of the Thesis

The aims of this study were to investigate the following for donkey hoof horn:

- To establish protocols for the collection, preparation and storage of hoof horn samples;

- To establish a protocol for the assessment of moisture content of hoof horn;

- To investigate the moisture content and hydrated moisture content of full and partial hoof wall depth samples for both donkey and horse hoof horn;

- To compare the moisture content and hydrated moisture content results from donkey hoof horn with those from horse hoof horn;

- To identify the effect of different relative humidity environments on the moisture content of donkey hoof horn;

- To establish tubule density within the *Stratum medium* of donkey hoof horn;

- To study the effect of a range of levels of hydration on the mechanical properties of donkey hoof horn measured by 3 point bending for full and partial hoof wall depth samples;

- To investigate the inter-relationships between the quantitative results for moisture content, tubule density and mechanical properties of donkey hoof horn.

2. ANALYSIS OF MOISTURE CONTENT OF THE *STRATUM MEDIUM* OF DONKEY HOOF HORN

2.1 Introduction

The moisture content of donkey hoof horn has not been reported. As reviewed in section 1.7, the collection, storage and preparation of hoof horn are very important when using samples of hoof horn for subsequent analyses, especially for the analysis of moisture content. Therefore the time taken between removal of clippings from the animal and their storage was examined and forms part of this present chapter.

From section 1.8 it can be seen that the sampling site and specific sample position is also likely to dictate the moisture content of hoof horn. This has been taken into account in sampling protocols in the present study by choosing a midline dead centre sample site on samples taken from clippings. The effects of age, gender and hoof pigment on the moisture content of hoof horn were not considered as part of the experimental design but are reported as interesting observations.

The difficulties in the determination of moisture content have also been outlined in section 1.8. These difficulties indicated that a standard method should be used in the actual determination of moisture content of hoof horn. The method used to establish moisture content in the present study was the same as that used by Von Bergen (1963). Moisture regain was also calculated to allow comparison with previous studies.

Many methods are available for the determination of moisture content, some of which have been used previously to ascertain the moisture content of hoof horn. Some of these different techniques are discussed.

2.1.1 Determination of Moisture Content

Indirect methods (section 1.8.3.3) were used in this project for the determination of the moisture content of donkey hoof horn. Examples of indirect determination of moisture content from samples are outlined below.

2.1.1.1 ELECTRICAL CONDUCTANCE

The use of electrical conductance is also an indirect method and has been used to assess moisture content in cattle hoof (Fuchs 1976; Martens 1975 and Müller 1976). Martens (1975) believed that this method was better than gravimetric methods as it produced a moisture content which took into account water that was chemically bound or was a fixed constituent of the microarchitecture of the hoof. Leuenberger *et al* (1978) believed that these results were not reproducible. These techniques were not therefore used in this study but would be an area for future investigation.

2.1.1.2 OVEN DRYING

Oven drying results in an increase in the partial vapour pressure of water in the solid which speeds up the rate of drying (Willits 1951). In wood science and technology, this is carried out at between 101°C to 105°C and is the standard method of determining moisture content (Pratt 1986). An oven temperature of 105°C is also generally used for the determination of the moisture contents of paper, board and wool (BS 2782 1991; BSEN 20287 1994; ASTM D1576-90 1995) demonstrating that standard techniques have been adopted in other areas.

During oven drying, energy in the form of heat is transferred from the air to the surface, raising the temperature of the sample together with the water it contains. Water, in the form of vapour, is then lost to the surrounding unsaturated air leading to a moisture content gradient from the inside to the outside of the sample. Raising the temperature will increase the steepness of this moisture gradient and also the rate of moisture movement along the gradient and thus the rate of loss of water vapour from the surface of the sample (Pratt 1986). However, high temperatures may cause sample volatilisation or decomposition (Willits 1951; ASTM D1576-90 1995). Hinterhofer (1996) used thermogravimetric analysis to analyse the stability of hoof horn at high temperatures and noted that samples did not visibly decompose until a temperature of 200°C was reached. It was therefore concluded that temperatures up to this level could be used to dry samples without degradation. Hinterhofer (1996) then chose 110°C for drying of samples of hoof horn.

Drying temperatures for hoof horn reported in the literature have ranged from 90°C to 115°C (Table 2.1). Different drying times have also been used. It is likely that both of these factors, together with the different methods for calculating moisture contents, will have resulted in differing moisture contents for hoof horn. In particular, an increase in temperature will cause the moisture gradient to increase, resulting in an increased rate of moisture loss from the surface of the sample.

An investigation into the effect of using different oven drying temperatures on the moisture content of donkey hoof horn was carried out in this present study. The range of temperatures used was 90°C to 120°C.

Bertram and Gosline (1987) believed that oven drying of hoof samples may cause a loss of very tightly bound water (Bertram and Gosline 1987). However, this was disputed previously for wool by Menefee and Yee (1965) who stated that water strongly bound to the protein chain is not removed until temperatures of 150°C. It is therefore unlikely in this present study that this type of water would be removed from samples within the temperature range to be tested.

Bergsten and Pettersson (1992) dried cattle hoof horn by placing it in a microwave oven. This method was not used in this study as the sample temperature could not be monitored.

Table 2.1 - Methods Used to Dry Equine Hoof Horn

Author	Hoof Wall Position	Percentage Moisture Content	Drying Method
Zschokke (1885)	Proximal wall Distal wall	28.8 28.5	110°C for several days
Smith (1887, cited in Smith 1921)	Wall	20.0	-
Thary (1896), cited in Butler 1976)	Wall	16.1	-
Clement (cited in Caulton Reeks 1905)	-	16.1	-
Smith (1921)	Wall	25.0	-
Gramatzki (1938, cited in Butler 1976)	Wall	24.6	-
Sassen (1938)	-	25.0	105°C
Benedetti (1948)	Wall	36.3	90-100°C until constant mass
Miyaki *et al* (1974)	Distal clippings	27.1	105-110°C - 10 hours. Desiccator 30 mins then reweighed.
Butler (1976) Butler and Hintz (1977)	Sole border Mid toe Coronary border	27.1 27.8 29.1	100°C - 7 days
Leach (1980)	Outer wall Inner wall	20.0 27.6	? 60°C - 5 days, 26-28 pounds vacuum
Douglas *et al* (1996), Douglas (1998)	Inner wall (n=24) Outer wall (n=24) Medial & lateral quarters	35.5 27.9 32.5	103.5°C until constant mass
Spitzlei (1996)	Distal clippings (3cm)	28.0	105°C - 24 hours
Hinterhofer *et al* (1998)	Dorsal and lateral wall	22.0	110°C - 50 hours
Ley *et al* (1998)	Clippings - toe and heel areas	31.2-33.8	115°C - 24 hours
Reilly (1999)	Dorsal Wall	3.5-33.9	Room temperature

2.1.1.3 Room Temperature Drying

An easy method of drying samples is by an air-dry basis at room temperature but this is generally used for *non*-hygroscopic substances which are samples that do not tend to absorb moisture strongly. Jackson (1992) suggested equilibration of samples at ambient conditions. By this means, Reilly (1999) dried hoof horn samples at room temperature to assess moisture content but his results indicated a great range of moisture contents for the full data set from 3.5% to 33.9%. This may be because drying would depend on the temperature, humidity and air movement within the room. This could easily vary from hour to hour. The conditions would therefore prove difficult to replicate if a comparison of moisture contents was needed. Drying at room temperature does avoid heating of the sample and therefore avoids the possibility of volatilisation or decomposition during heating (Willits 1951).

2.1.1.4 Chemical Methods

A common chemical method of indirect determination is Karl Fischer analysis which involves the use of a relatively specific reagent for water (Skoog and West 1976). Preparation would involve grinding of the sample thereby causing moisture loss which would, in effect, defeat the object of moisture content assessment.

Another chemical method is the use of desiccants which increase the drying rate by increasing the water vapour pressure differential as a result of lowering vapour pressure in air (Willits 1951). Phosphorus pentoxide is regarded widely as a most efficient desiccant (residual water 0.0002 mg/l of air) and was used by Bertram and Gosline (1987) to dehydrate their horse hoof samples prior to mechanical testing.

2.1.1.5 Vacuum Drying

Another method of dehydration that results in increasing the drying rate by increasing the water vapour differential is the use of vacuum drying. This method is a common approach for determining the moisture content of wool (Le Compte and Lipp 1949) and has been used to assess hoof moisture contents by Leach (1980). However, the temperature was also raised to 60°C.

2.1.1.6 FREEZE-DRYING

Freeze-drying is generally used to preserve biological materials. The sample is initially frozen, generally under vacuum, by contact with a cold air current or refrigerated plate. Ice crystals are formed within the sample. Heat is supplied to the sample causing the ice to sublime which is a change of state from ice to vapour without melting. The vapour given off is prevented from returning via vacuum and a cold trap or condenser. Secondary drying is carried out at approximately 30°C for biological materials (Mellor 1978).

There are some advantages of using freeze-drying methods. Samples generally have an open porous structure following freeze-drying that facilitates reabsorption of water (Nonhebel and Moss 1971). The use of freeze-drying also avoids possible loss of other components which may evaporate with the water at higher temperatures (Nonhebel and Moss 1971) and also avoids denaturation of proteins (Mellor 1978). This method is not believed to have been reported for drying hoof samples.

2.1.2 Selection of Methods Used in the Present Study

As oven drying seemed to be the method preferred by many authors to dehydrate samples to assess moisture content, oven drying of samples at different temperatures between 90°C and 120°C was used in this present study.

Phosphorus pentoxide was also used in this study as a desiccant as it avoids the possible effects of sample damage by high temperatures. Bertram and Gosline (1987) had previously used this desiccant to dry samples of hoof horn prior to mechanical testing.

Room temperature drying was also used for comparative purposes during this present study as this had been used by Reilly (1999). Interpretation of the results must take into account the fact that this method does not allow for control of the relative humidity or air movement surrounding the sample.

It was decided to use vacuum drying as one of the test dehydration methods in this thesis. This method was not, however, combined with an increased temperature. Leach (1980) had used a temperature of 60°C during vacuum drying of hoof samples.

Although freeze-drying has not been used by other authors to determine the moisture content of hoof horn, it was decided to use this method following assessment of the advantages of freeze-drying (section 2.1.1.6).

Real progress in this field will not be achieved until standardised practices are developed and accepted. A standardised method must be established in future work to determine hoof moisture content. A number of steps must be considered to achieve this aim. These include sample collection, storage and preparation which were discussed in section 1.7 and, finally, the actual method of determining hoof moisture content. This final step for the determination of moisture content is the subject of this part of the study and aims to assess whether different methods of dehydrating hoof samples result in differing moisture contents and to assess the time involved for sample equilibrium mass to be achieved during drying. A standard protocol was then established for the analysis of the moisture content of hoof horn. The method chosen as the standard was then used to ascertain the moisture content of partial HWD samples.

In addition, the quantitative analysis of moisture content of donkey hoof horn needs to be obtained in order to investigate the inter-relationships with other quantifiable characteristics of hoof horn. This will enable samples to be tested at physiological moisture levels, thus representing the *in vivo* condition (Douglas *et al* 1996; Hinterhofer 1996; Hinterhofer *et al* 1998). This will then aid in the understanding of the function of the water content of hoof horn.

As no direct comparisons of the moisture content of donkey hoof horn could be made with those from horse hoof horn from the literature, owing to different methodologies being used, samples of clippings from horse hoof were also examined by the same technique for comparative purposes.

2.2 Aims

The aims of this part of the study were:

- To investigate whether the time between removal of clippings from the animal and their storage was important with regard to maintaining an *in vivo* moisture content;

- To assess whether different methods of dehydrating hoof samples result in different hoof horn moisture contents;

- To propose one method to be used as a standard technique for future work to determine the moisture content of donkey hoof horn;

- To assess optimum drying times for the different methods of dehydrating hoof samples;

- To use the proposed method of dehydration to determine the moisture content of donkey and horse hoof horn.

2.3 Materials and Methods

2.3.1 Donkeys

A population of thirty one donkeys, which comprised eleven females and twenty males, was selected from those at The Donkey Sanctuary, Sidmouth, Devon. The animals were aged nine and under (Table 2.2).

Table 2.2 - Summary Table of Donkey Population Used for Clipping Samples

Age at Start of Project (years)	Number of Donkeys
3	3
4	2
5	16
6	4
7	3
8	2
9	1
Total	31

Their history showed that thirteen of these donkeys had been born at The Donkey Sanctuary, and the remainder had been living there for more than one year but the history of the individuals prior to moving to The Donkey Sanctuary was unknown. Details of all donkeys are outlined in Table 2.3. Their bodyweights at the start of the study were provided by The Donkey Sanctuary and had been taken from the results of a regular monthly check at the start of the project using a portable weighbridge. Animal bodyweights were recorded to see if there was any relationship between tubule density and bodyweight.

All of these donkeys were kept on the same farm. All animals were wormed regularly, had their teeth rasped and their feet were trimmed every ten weeks as far as was reasonably possible, by the same farrier. During the winter the donkeys were loose-housed in a barn on straw bedding with access to concrete yards. They were

fed straw from communal mangers. The donkeys were kept at grass during the summer. There was access to *ad lib* water.

Table 2.3 - Details of Donkey Population Used for Collection of Hoof Clippings

Donkey	Age at Start of Project (years)	Gender - Male (M) Female (F)	Bodyweight at Start of Project (kg)	Farrier Group	Pigmented (P), *Non*-pigmented Hoof (NP)
1	5	M	160	3	P
2	5	F	175	1	P
3	5	M	150	3	NP
4	5	F	175	2	P
5	5	F	125	3	P
6	5	M	255	3	P
7	4	M	150	3	P
8	7	F	185	2	P
9	5	M	170	2	P
10	5	F	145	3	P
11	3	F	135	1	P
12	3	F	145	1	P
13	3	M	145	1	P
14	5	M	185	3	P
15	5	M	175	1	P
16	5	M	185	1	NP
17	5	M	175	1	P
18	5	F	210	1	NP
19	5	M	150	1	P
20	5	F	165	1	NP
21	8	M	205	1	NP
22	4	M	290	2	P
23	5	M	200	2	NP
24	7	M	210	2	P
25	6	F	220	2	P
26	7	M	215	2	P
27	6	M	165	2	P
28	6	M	225	3	NP
29	6	M	150	3	P
30	8	M	260	2	P
31	9	F	210	2	P
Median* Mean**	5*	-	184**	-	-
Standard Deviation	-	-	39	-	-

The group was divided into three for farriery purposes (Table 2.3). The hooves of the three groups were trimmed to produce hoof clippings at different times over a period of three weeks. There was no medical history of problems associated with their feet during their stay at The Donkey Sanctuary prior to the commencement of the project. Samples from the fore limbs only were used throughout the study as some animals tend to drag their hind feet and wear away the toe area. There were, however, problems with some of the donkeys' feet during the project which were diagnosed by The Donkey Sanctuary veterinarians and farrier as seedy toe and white line disease. These problems cause physical damage to the white line and the hoof wall itself by invasion of bacteria and/or fungi. Samples from affected animals were not used for analyses. Five donkeys left the trial group as they were found private homes during the trial period. These animals were not used for further sampling. From the hooves of the animals used, seven were *non*-pigmented and the remainder were pigmented (Table 2.3).

2.3.2 Horses

A population of sixteen horses which comprised five females and eleven males was selected from those at the Friends of Bristol Horses Society, Bristol. The age of the animals ranged from 12-34. The animals were not shod and had their feet trimmed regularly. Details of the horses are provided in Table 2.4.

2.3.3 Preliminary Experiments for Assessment of Moisture Loss from Hoof Horn

Two preliminary experiments were carried out that involved monitoring the mass loss of samples over time following removal from the animal.

The farrier removed clippings from the left fore of five of the trial donkeys (Appendix 1) using sharp hoof cutters to prevent tearing of the sample. The left fore limb was chosen arbitrarily and was used throughout this study. The removal of the clipping was started at the medial or lateral heel rather than at the toe region of the

hoof in order to produce a full clipping and to avoid damaging the toe area of the clipping.

Table 2.4 - Details of the Horse Population Used for Collection of Clippings

Horse	Age at Start of Project (years)	Gender - Male (M) Female (F)
1	31	M
2	19	M
3	20	M
4	12	M
5	15	F
6	24	M
7	23	F
8	21	M
9	23	M
10	21	F
11	34	M
12	26	F
13	26	F
14	30	M
15	20	F
16	18	M

The area of individual clippings to be used for subsequent analyses was taken from the MDC region after Reilly *et al* (1996) and is indicated in Figure 2.1. The method was adapted for the clipping in the following way. The sample was laid on a piece of paper and an inverted "V" shape was drawn where it was estimated the frog would be if the clipping was still attached to the hoof. A line was drawn to bisect the frog. The intersection of this line with the hoof wall was called the MDC.

Preliminary work prior to this project (Hopegood, L. unpublished observations) showed that hoof horn samples that were wrapped in three layers of Parafilm (an arbitrary number) and stored at 4°C for three months lost a mean of 0.55% of their mass. This mass loss following storage was assumed to be equivalent to the loss of moisture content. This therefore indicated that only a very small percentage of moisture had been lost from the samples. Parafilm moulds easily to the shape of the

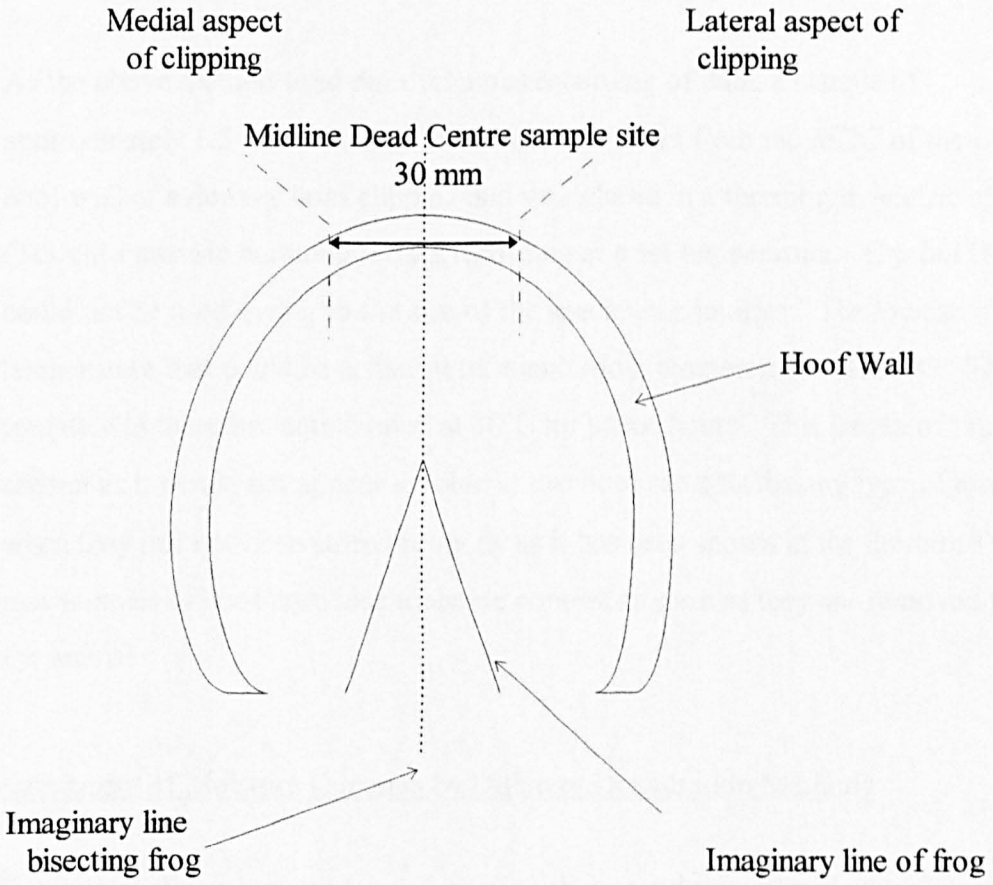
sample and can be made to form an airtight seal by overlapping the edges on each turn and pulling it taut during application. This also avoids the problem of headspace being present.

Parafilm was used in preference to plastic bags as they are generally made from a film of 0.025 mm whereas the thickness of Parafilm is 0.125 mm. Parafilm possesses a permeability of 3-4g/m^2/day/0.125 mm at 38°C and 80% relative humidity compared to the much higher permeability of clingfilm. The overall permeability of plastic bags is also higher than for Parafilm. Parafilm was therefore used as the method of storage of hoof horn for this project.

Therefore, the method used in this thesis to conserve the moisture content of hoof horn samples was by wrapping them in three layers of Parafilm. The method used here was summarised by Collins *et al* (1998) who used Parafilm to wrap samples prior to analysing donkey hoof horn for moisture content. The following experiments were to identify the timescales involved by which samples of hoof horn needed to be stored to avoid moisture loss.

Figure 2.1 - Position of Sample Site on Left Fore Limb Hoof Clipping

(As seen from the solear aspect of the hoof. Not to scale.)



The five samples taken were wrapped immediately on removal from the animal. Samples were then labelled according to the hoof from which they were removed by writing with waterproof overhead projector pens directly onto the Parafilm. Samples were placed in plastic bags and labelled with the animal's name and sampling date and then they were stored at 4°C for one week.

Samples were unwrapped as they were needed. The MDC sample was removed from the clippings using a Stanley knife. The section removed was 30 mm in length, with 15 mm either side of the MDC. The white line was removed using a scalpel. One sample was removed from each MDC and encompassed the full HWD and was approximately 1.5 mm wide and 1.5 mm in depth. Samples were weighed to provide an original *in vivo* mass and were then kept at room temperature (23°C). Samples

were reweighed and their mass recorded every five minutes over a period of sixty minutes. Moisture contents were calculated as a percentage of the original mass (Equation 1). This was taken to be equivalent to an *in vivo* moisture content.

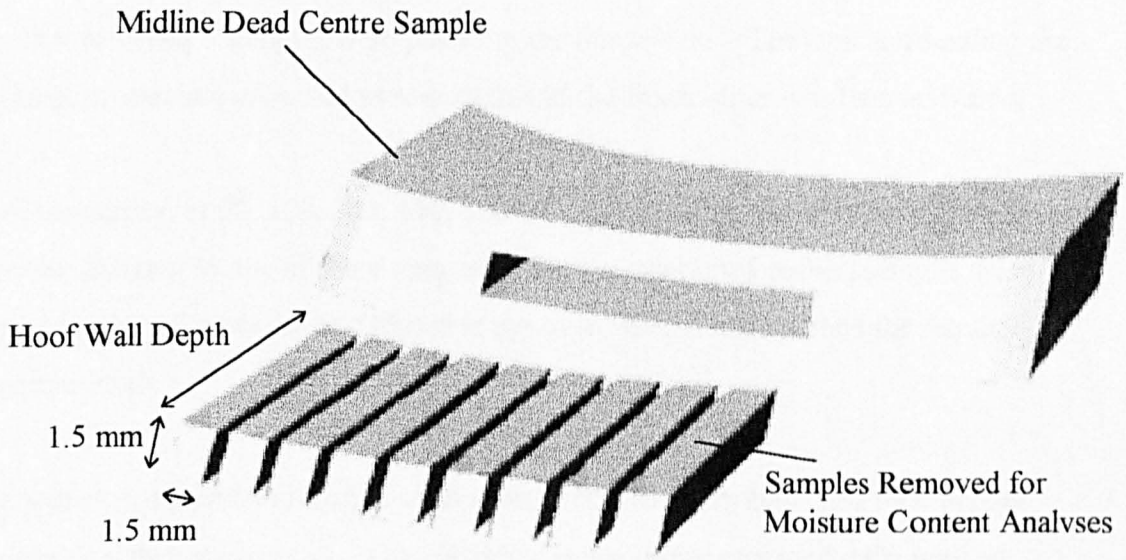
As the above method used discontinuous recording of data, a sample of approximately 1.5 mm x 1.5 mm x 1.5 mm was taken from the MDC of the outer hoof wall of a donkey hoof clipping and was placed in a thermogravimetric analyser[2] (TGA) to provide continuous data recording at a set temperature. The full HWD could not be used owing to the size of the specimen container. The lowest temperature that could be achieved to mimic room temperature was 30°C. The sample was therefore equilibrated at 30°C for seven hours. This length of time was chosen as it would not appear feasible to use hoof samples for any type of analysis when they had not been stored properly as it has been shown in the literature review that samples of hoof horn lose moisture content as soon as they are removed from the animal.

2.3.4 Assessment of Moisture Contents by Different Dehydration Methods

Samples of clippings were taken following the procedure outlined above from the left fore of all thirty one donkeys (Appendix 1). Samples were unwrapped and the MDC section of 30 mm in length was removed and divided about the MDC into nine samples across the full width of the hoof wall using a scalpel. Samples were exposed for approximately forty five seconds. Samples were then wrapped in Parafilm to maintain moisture content. The sample sizes were approximately 1.5 mm x 1.5 mm x HWD (Figure 2.2).

2 Thermoanalyzer 50, TA Instruments

Figure 2.2 - Diagram Showing Midline Dead Centre Sample Divided into Samples for Moisture Content Analyses



After preparation, samples were weighed immediately following removal from the Parafilm. This established their fresh mass. One sample from each animal was then allocated randomly to each of the individual drying methods. This meant that each drying environment had thirty one samples. These were contained in six small, unsealed glass bottles with a maximum of five samples in each bottle. Due to the odd number of samples involved one bottle contained six samples. Samples were identified using different coloured dots made from indelible markers. Samples for oven drying were placed in crucibles. Samples were unwrapped and placed in their respective environments as quickly as possible:-

- Room temperature - the bottles were placed in the laboratory, the temperature of which was approximately 23°C;

- Drying under vacuum at room temperature - samples were placed in a desiccator and a vacuum applied;

- Drying over phosphorus pentoxide - samples were placed in a desiccator on a shelf of wire gauze over the top of a layer of phosphorus pentoxide;

- Freeze-drying - samples were placed in the freeze-drier. The area surrounding the samples was then subjected to a vacuum and the freeze-drier was then activated;

- Oven drying at 90, 100, 105, 110, 120°C - the remaining five groups of samples were allocated to one of these temperatures. As mentioned earlier, samples were placed in small crucibles and placed in the oven when it had reached the required temperature.

Samples were removed from their environments on a daily basis and their masses were weighed immediately. The individual masses were recorded daily until an equilibrium mass was reached (Jackson 1992). The time taken for equilibrium mass to be achieved was decided following statistical analyses of the daily recorded masses over time (Mann-Whitney *U* tests). The final moisture content was expressed as a percentage of original mass. Moisture content was used as this represented a physiological value which can be related back to the original and *in vivo* mass.

Comparisons were made between the final moisture contents for the individual environments. Data for the results from the phosphorus pentoxide drying regime were analysed to see if there was a difference in moisture content between males and females for pigmented and *non*-pigmented hoof and also to see if animal age influenced moisture content.

Moisture regain was calculated using Equation 2 for samples dried over phosphorus pentoxide only to provide results to compare with those from other authors.

2.3.5 Moisture Content of Horse Hoof Horn

The MDC of samples was used from the clippings of the left fore of the sixteen horses detailed in Table 2.4. The protocol for collection and preparation of samples was as outlined in section 2.3.3 for donkey hoof clippings. However, only one sample was needed. This was removed from the MDC with dimensions of 1.5 mm x 1.5 mm by the HWD. Samples were dehydrated by placing them over phosphorus pentoxide. Both the moisture content and moisture regain were calculated.

2.3.6 Statistical Analyses

Throughout the thesis the data were analysed using Minitab[3]. Initial assessments of data were carried out to see if the data sets possessed a normal distribution. The level of significance was taken as $p<0.05$ unless otherwise stated. The parametric tests used include: one way analysis of variance (ANOVA), Tukey test and Student's *t* test. The *non*-parametric tests used were the Kruskal-Wallis and the Mann-Whitney *U* tests. Assessments using correlation and regression tests were also carried out.

2.4 **Results**

2.4.1 The Effect of Delay of Storage on the Moisture Content of Hoof Horn

Figure 2.3 shows the mean mass loss of samples as a percentage of the original mass at the end of each five minute period following manual weighing of samples resulting in a 21% loss after one hour.

Figure 2.4 indicates the mass loss obtained by the thermogravimetric analyser of the outer hoof wall of the donkey hoof sample equilibrated at 30°C for seven hours. This showed a mass loss of approximately 14% of the original mass over the first hour and then a total mass loss of 16% over the seven hours.

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3 Minitab™ version 13.31, Minitab Inc., USA

Figure 2.3 - Drying of Donkey Hoof Clippings at Room Temperature (n=5)

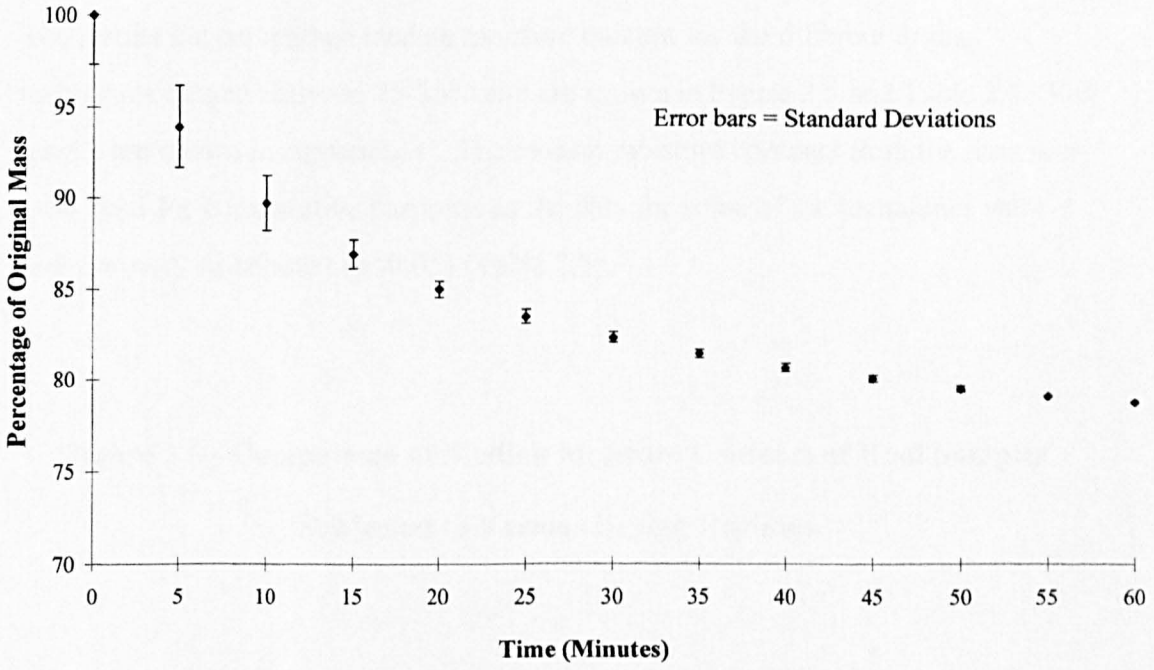
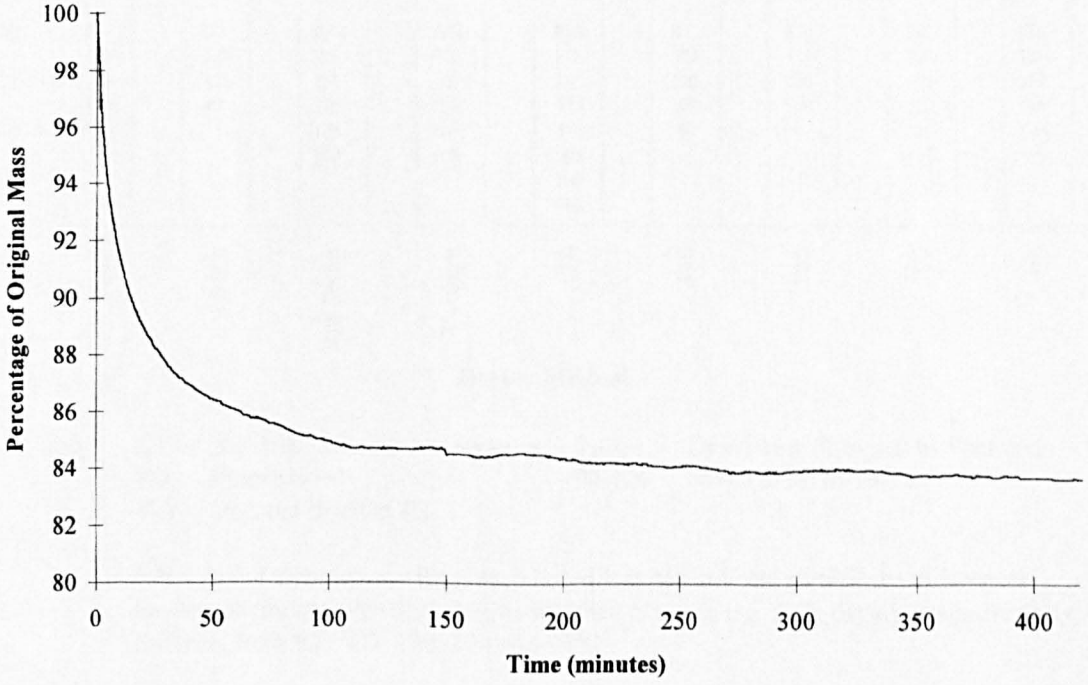


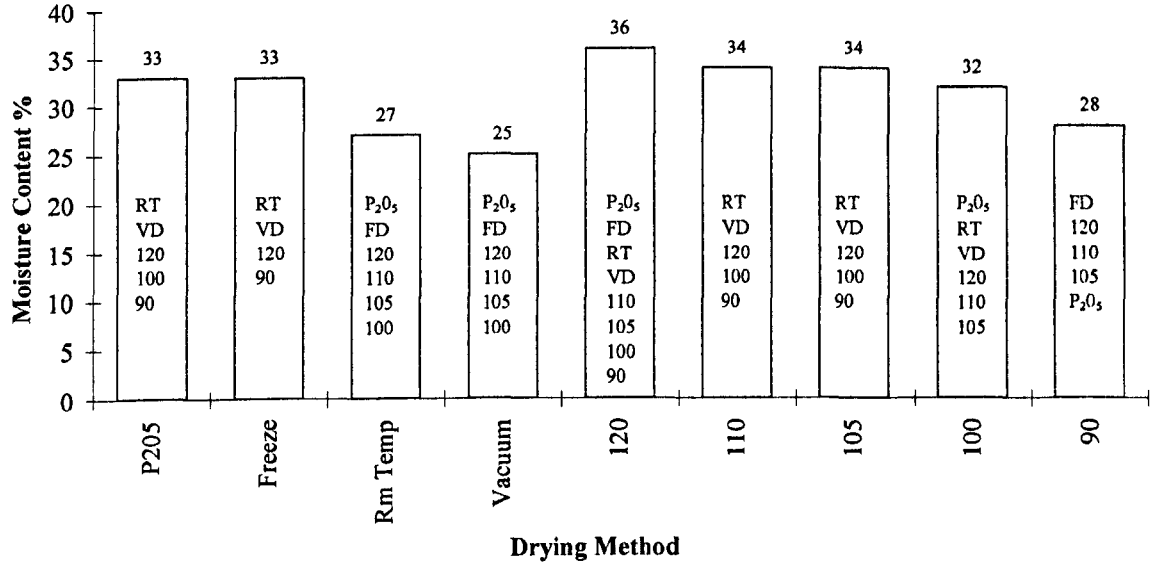
Figure 2.4 - Mass Loss of Donkey Hoof Sample Equilibrated at 30°C for 7 Hours (n=1)



2.4.2 Comparison of Different Techniques to Assess the Moisture Content of Hoof Horn

The results for percentage median moisture content for the different drying techniques ranged between 25-36% and are shown in Figure 2.5 and Table 2.5. Full results are shown in Appendix 1. The median moisture contents from the data sets were used for comparative purposes as the data for some of the techniques were *non*-normally distributed ($p<0.05$) (Table 2.5).

Figure 2.5 - Comparison of Median Moisture Contents of Hoof Samples Subjected to Various Drying Regimes



Key:

RT	Air dried at room temperature	P_2O_5	Dried over Phosphorus Pentoxide
FD	Freeze dried	90-120	Oven dried at 90-120°C
VD	Vacuum dried at RT		

NB: Lists of techniques within each individual bar indicate significant differences between techniques ($p<0.05$, Mann-Whitney *U* tests), *e.g.* P_2O_5 drying is significantly different from RT, VD, 120, 100 and 90°C.

Table 2.5 - Summary of Moisture Content Results from Different Drying Techniques

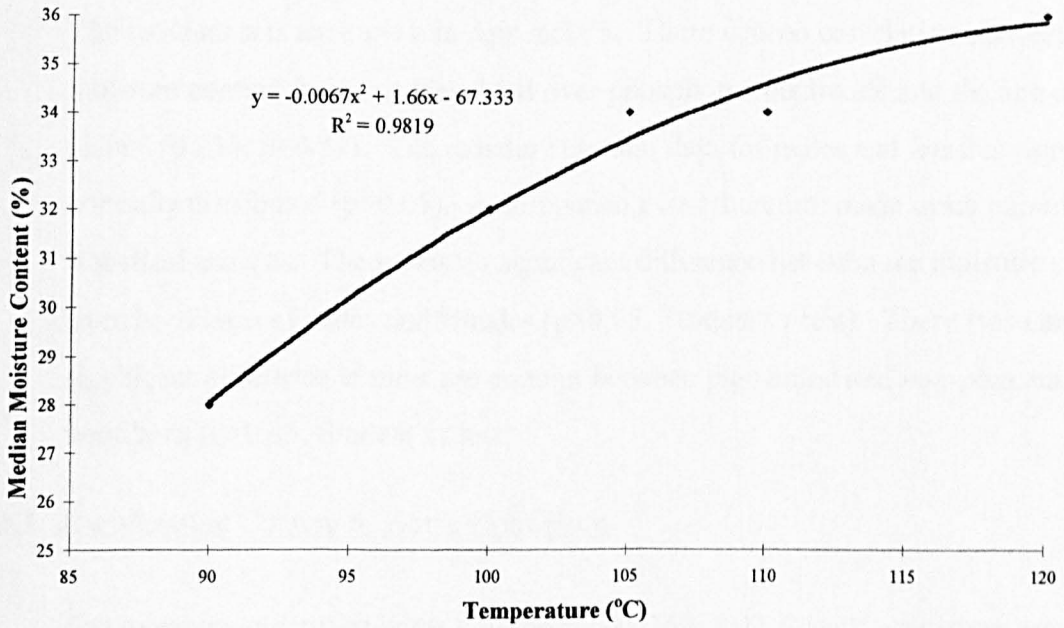
Drying Method	P_2O_5	Freeze Drying	Room Temp'	Vacuum Drying	120°C	110°C	105°C	100°C	90°C
Mean (%)	33	33	26	23	36	34	33	29	27
Median (%)	33	33	27	25	36	34	34	32	28
Standard Deviation	2	3	N/A	N/A	2	3	N/A	N/A	N/A
Coefficient of Variation (%)	6	8	14	44	4	7	18	25	28
Range (%)	29-37	26-38	14-31	6-36	32-39	27-38	11-39	10-37	10-38
P-value	0.086	0.837	0.002	0.000	0.514	0.058	0.000	0.000	0.000
Normally Distributed? (p>0.05)	Yes	Yes	No	No	Yes	Yes	No	No	No

The median moisture content of donkey hoof horn determined from combining results from drying samples by all techniques was 33%. A Kruskal-Wallis test initially showed there were significant differences between medians for the results of the different drying methods. The results from each technique were significantly different to those from at least four other methods ($p<0.05$, Mann-Whitney *U* tests) (Figure 2.5). The median moisture content of 36% for samples dried at 120°C was significantly different from all other methods ($p<0.05$, Mann-Whitney *U* tests). Although both drying over P_2O_5 and freeze-drying resulted in the same median moisture contents of 33%, the results for freeze-dried samples were not significantly different from samples dried at 100°C ($p=0.0574$), whereas the results of samples dried over P_2O_5 and those dried at 100°C were significantly different ($p=0.0426$).

The mean moisture regain for samples of donkey hoof horn dried over P_2O_5 was 50% (SD 5, CV 10%).

An increase in drying temperature for oven dried samples generally showed an increase in sample mass loss and resultant difference in moisture content (Figure 2.6).

Figure 2.6 - Median Moisture Contents of Donkey Hoof Horn When Dried at Different Temperatures (the second order polynomial was fitted for ease of reference only)



There was a significant positive correlation (0.84) between oven temperature and median moisture content ($p=0.038$). A regression analysis of the data resulted in an R^2 value of 70% ($p<0.05$). The regression equation was:

% Median Moisture Content = 24 + 0.0862 Temperature

2.4.2.1 DRYING TIMES

Oven dried samples were dried for five days although equilibrium mass was achieved after one day as there was no significant difference between the moisture contents determined at day 1 and day 5 ($p>0.05$, Mann-Whitney *U* test). The samples dried at both room temperature and over phosphorus pentoxide were dried for nine days,

although constant mass was achieved after five days. The moisture content of samples dried by freeze drying reached an equilibrium value of 33% after five days. Samples dried by vacuum drying achieved equilibrium mass after three days.

2.4.2.2 The Effect of Age, Gender and Hoof Pigment on the Moisture Content of Donkey Hoof Horn

The full data sets are shown in Appendix 3. There was no correlation between moisture content from samples dried over phosphorus pentoxide and the age of the animal (0.234, $p=0.21$). The moisture content data for males and females were normally distributed ($p>0.05$). A comparison was therefore made using parametric statistical analysis. There was no significant difference between the moisture content from hoof horn of males and females ($p>0.05$, Student's *t* test). There was also no significant difference in moisture content between pigmented and *non*-pigmented hoof horn ($p>0.05$, Student's *t* test).

2.4.3 The Moisture Content of Horse Hoof Horn

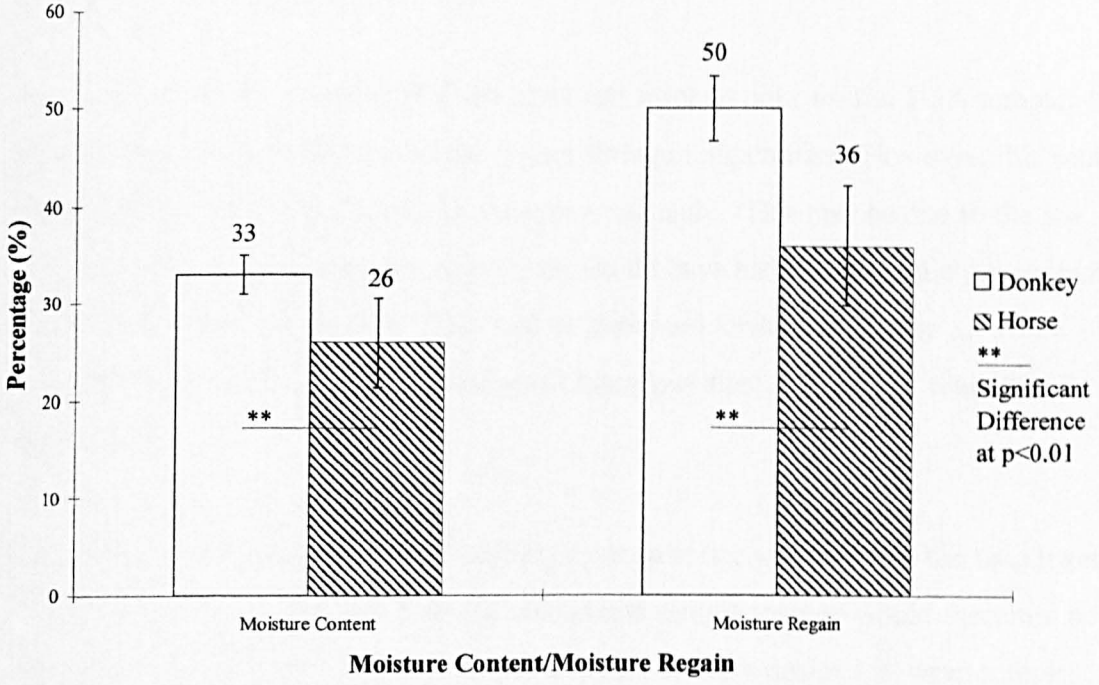
The moisture content of horse hoof horn was 26% (SD 3) with a moisture regain of 36% (SD 6). The full data set is shown in Appendix 4.

2.4.4 Comparison of the Moisture Content of Donkey and Horse Hoof Horn

Both the moisture content and moisture regain of donkey hoof horn were significantly higher than that of horse hoof horn ($p<0.01$, Student's *t* test) (Figure 2.7). The full data sets are presented in Appendix 4.

Some of the results from this chapter are outlined in Cope *et al* (1998) and Reilly *et al* 2002d).

Figure 2.7 - Comparison of Moisture Content and Moisture Regain of Donkey (n=31) and Horse Hoof Horn (n=16)



2.5 Discussion

The method of ascertaining the MDC of the clipping samples could be questioned as the method uses an "imaginary line bisecting an imaginary frog". The method of using a line to bisect the frog, be it an existing or an imaginary frog, may cause problems as very often the frog is not placed centrally, or is twisted, and therefore the line running along the centre of the frog does not bisect the whole hoof capsule. It may have been better to have marked the MDC of the samples prior to removal from the animal but

this would still not have helped if the frog was not located centrally. Until another method is designed this is believed to be satisfactory.

2.5.1 The Effect of Delay of Storage on the Moisture Content of Hoof Horn

Dehydration of both sets of samples showed a continuous mass loss over time. The results from the samples that were weighed manually showed a steep decline over the first fifteen minutes which was as a result of a high rate of mass loss which was equated to moisture content loss during this time.

It would have been expected that the mass loss after an hour for the TGA sample should have been greater due to the higher storage temperature. However, this was actually lower than those tested by weighing manually. This may be due to the use of an outer hoof wall specimen only which would have had a lower moisture content than the full HWD specimens. This will be explained further in Chapter 3. There was not a great deal of difference between mass loss after an hour and that after seven hours.

The results from both of these preliminary experiments confirmed that the time taken between removal of samples from the animal and sample storage would therefore be important for moisture content analyses. It was therefore decided to wrap samples in Parafilm immediately on removal from the animal.

The mass loss for both sets of results was higher than the two samples tested by Smith (1887) who noted that after twenty four hours two pieces of hoof had lost just 2.5% and 1.9% of their mass. This may have been due to a high humidity environment surrounding the samples. However, direct comparisons could not be made as sampling details and methodologies were not provided.

From the results presented, samples of hoof horn used for moisture content analyses must be protected against moisture loss as soon as they are removed from the animal.

The following protocol was used in subsequent work for the collection and storage of donkey hoof clippings:

- Full clippings to be removed by farrier with sharp hoof cutters to prevent tearing of the sample, starting at the medial or lateral heel;

- The sample should be wrapped immediately in three layers of Parafilm by wrapping the Parafilm around each sample, overlapping the edges each time to make an airtight seal and pulling it taut during application to mould it to the shape of the sample;

- Samples to be placed in plastic bags and labelled to include name, hoof, sample area (if it is not a full clipping), together with date the sample was taken. Samples to be stored at 4°C.

2.5.2 Comparison of Different Techniques to Assess the Moisture Content of Hoof Horn

The method of calculating moisture content as a percentage of fresh or original mass resulted in the median moisture contents shown for donkey hoof horn samples dried by the different techniques being between 25 and 36%. There were significant differences between the moisture content results of many of the techniques (Figure 2.5).

As there appeared to be no previous studies carried out on the moisture content of donkey hoof, direct comparisons with this study could not be made. Comparisons were therefore made with literature on horse hoof and then also with the results from horse hoof for samples dried over P_2O_5 in this present study. It is acknowledged, first and foremost, that many of the differences in moisture content reported may be as a result of a species difference between the moisture content of donkey and horse hoof.

As would be expected, the moisture contents determined by the oven drying techniques generally increased with an increase in temperature. This would be expected as mass loss occurs when moist samples are subjected to oven drying and there would be large temperature differentials between the warm air and the wet sample according to the temperature of the oven.

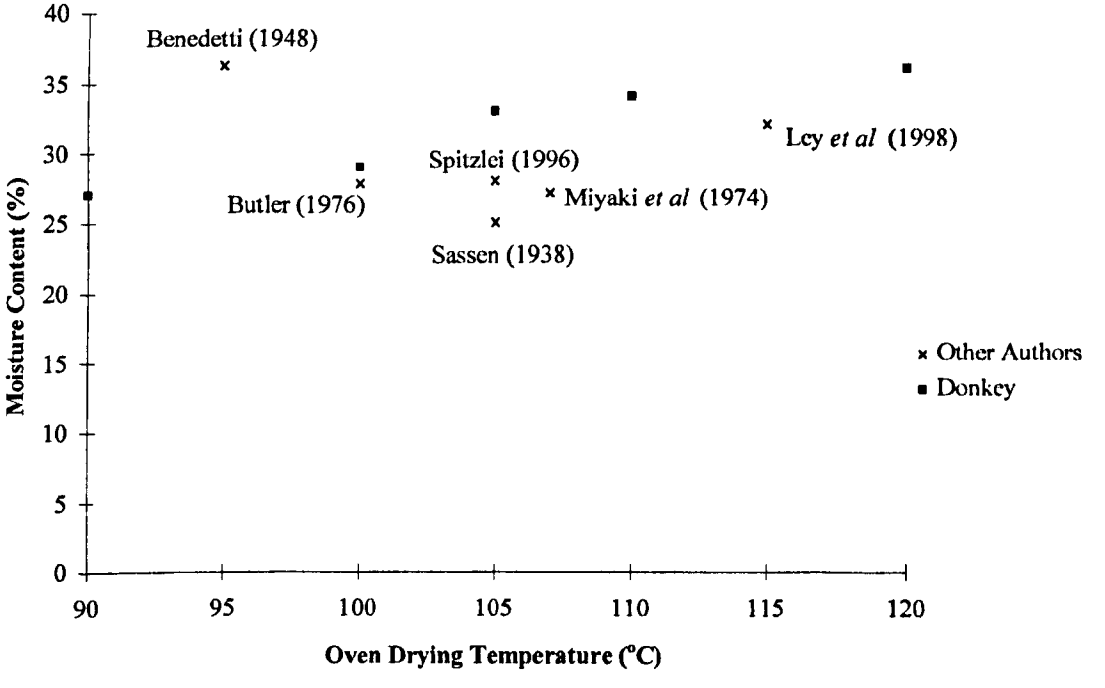
The results following drying of hoof at 90°C, 100°C and 105°C were *non*-normally distributed ($p<0.01$). However, results for those samples dried at 110°C and 120°C were normally distributed ($p>0.05$) and showed lower coefficients of variation (Table 2.5) which reflected the low degree of variability in the results for these samples compared to the results for the lower temperatures. This was probably due to the inefficiency of the lower temperatures in removing moisture from the samples. As has been previously mentioned, authors have used various temperatures of up to 110°C to dehydrate samples (Sassen 1938; Benedetti 1948; Butler 1976; Spitzlei 1996). Therefore it is likely that their data sets also showed a high variability. Either a high temperature or a different method should be adopted. It is interesting to note the significant difference of 6% in median moisture content between samples dried at 90°C and those dried at 105°C. If samples were subsequently to be tested mechanically, it is likely that this difference in moisture content would confer upon the samples a significant difference in mechanical properties. Therefore, results from mechanical testing following samples dried at these temperatures should not be compared.

Willits (1951) believed that high temperatures may cause sample volatilisation and decomposition. Another reason why high temperatures should be avoided is that certain proteins, on drying, may exhibit a partial or complete irreversible loss of biological activity and the voids produced by loss of water may result in strains in the molecules of dried proteins (Kuntz and Kauzmann 1974). As has been shown for wood, the outer layer of samples may tear or split if the moisture gradient becomes too steep (Pratt 1986). This may occur in hoof horn samples when using such high

temperatures to dry samples. These temperatures should be avoided if subsequent mechanical testing is to be carried out as a steep moisture gradient may damage the sample. Heating samples at elevated temperatures to remove moisture may therefore cause the mechanical properties of the material to change. Oven drying may not be the most appropriate technique to assess moisture content prior to mechanical testing, although this drying technique is less time consuming than some of the other methods. It has also been the most frequently used method of assessing hoof moisture contents. Some of the results for horse hoof horn will be compared to those from the present study. It must, however, be borne in mind that any differences shown may be as a true difference between the two species.

The results from other authors of dehydrating samples of hoof horn by using oven drying techniques are shown in Figure 2.8. As there is no uniform increase in moisture content with an increase in temperature, their individual protocols together with their results must be questioned.

Figure 2.8 - Previously Published Moisture Content Results for Full Hoof Wall Depth Samples of Horse Hoof Horn Compared with Donkey Hoof Horn Following Drying at Different Temperatures



The early work of Sassen (1938) established a moisture content of 25% for horse hoof following drying at 105°C. This was considerably lower than the 34% found at the same temperature for donkey hoof horn in this study. Sampling protocols may explain the differences but Sassen (1938) did not describe his sampling methodologies.

Benedetti (1948) found a moisture content of 36% with samples that were oven dried at 90-100°C. This was much higher than for donkey hoof horn and actually equated to the moisture content results of samples dried at 120°C. The use of morbid samples and different sampling sites in this instance may be the reason for the differences.

Bertram (1984) dried samples at 80°C for three days and found that drying beyond three days produced no change in weight. Butler (1976) noted that hoof lost moisture until day 5 or 6 when drying in an oven at 100°C. This is contrary to the findings of the present study as although samples were dried for three days, there was no significant difference in the results between those dried for one day and those dried for three days. A direct comparison could not, however, be made owing to differences in sampling methodologies.

Butler and Hintz (1977) reported that the moisture content for the wall at the sole border was 27.1% following oven drying at 100°C for seven days. The same temperature used in the present study resulted in a higher moisture content of 32%. It is likely that the timings of sample collection, together with the method of storage may be responsible for these differences as the authors used morbid hooves and did not provide detailed explanations of protocols. They may not have realised the necessity of collection of samples as soon as possible after euthanasia.

Miyaki *et al* (1974) examined hoof clippings with samples taken within 5 mm from the edge of the hoof which had a moisture content of 27.1% following drying at 105-

110°C for ten hours. Again, this is lower than the 34% achieved at the same drying temperature after twenty four hours in these experiments. Again, sample collection, drying time or area of hoof sampled may account for the difference.

Moisture content analyses on horse hoof distal clippings resulted in moisture contents of 27.1%, 27.1% and 28% respectively (Miyaki *et al* 1974; Butler and Hintz 1977; Spitzlei 1996). These are lower than those found in the present study and may indicate a species difference between horse and donkey hoof horn. The later work of Ley *et al* (1998) used a temperature of 115°C and found a moisture content of 31.16-33.83% for horse hoof. This higher result may be attributable to the use of clippings from all four feet together with the use of heel samples which, as previously explained, may possess a higher moisture content.

2.5.2.2 FREEZE-DRYING

A direct comparison of these present results could not be carried out as there were no previously reported uses of freeze-drying to determine the moisture content of hoof horn.

However, drying of samples by freeze-drying may be a suitable technique as it avoids the effects of high temperatures; the resultant data were normally distributed ($p>0.05$) and showed low variability. The influence of freeze drying on the mechanical properties of hoof has yet to be elucidated as this technique has not been used before. One advantage of freeze-drying is that water reabsorption into freeze-dried samples should be facilitated by samples maintaining an open porous structure (Nonhebel and Moss 1971). This would be useful during rehydrating samples to attain an *in vivo* moisture content.

2.5.2.3 DRYING AT ROOM TEMPERATURE

The data set of median moisture contents for samples dried at room temperature of 27% was *non*-normally distributed ($p<0.01$) and was much lower than six other techniques. This reflects only the slight differential between the environmental

temperature and that of the sample as only a small amount of moisture has been removed. It must also be appreciated that only the dorsal wall and sole *in vivo* are in contact with the surrounding environment. In this experiment it is possible to lose moisture from all six surfaces of the sample.

There was no significant difference between the median moisture content for both treatment and control groups (n=4 in both groups) for pony hoof horn (Reilly 1999) ($p>0.05$). The data were then combined, resulting in a median moisture content of 24% which is slightly less than the 27% shown for donkey hoof horn. This slight difference may be due to differences in sample preparation as Reilly (1999) machined samples for mechanical testing whereas the samples of donkey hoof horn were prepared using a knife. The combined data set from Reilly (1999) was then compared to the results of the donkey hoof horn samples that were also dried at room temperature. There was no significant difference between the two data sets ($p=0.058$) (Mann-Whitney *U* test), indicating that there is no difference between the median moisture content of both donkey and horse hoof horn. Large variations were, however, seen in both data sets, with pony hoof horn showing moisture contents between 13-29% and donkey hoof horn showing a range of between 14-31%. These large ranges for both data sets may be explained by the lack of environmental control during drying at room temperature.

Although drying samples at room temperature avoids sample heating, it is important that the environment in which the sample is dried is controlled, otherwise factors such as temperature, relative humidity and air movement will affect the moisture content of the samples.

2.5.2.4 Vacuum Drying

The moisture content results from vacuum dried samples were even lower than those dried at room temperature and also showed a *non*-normal distribution. Again, this method avoided sample heating but the low results reflect the need to control for

relative humidity entering the environment to avoid the sample gaining moisture in this way. The air entering should be dried prior to being passed over the sample.

2.5.2.5 Drying Over Phosphorus Pentoxide

Drying of samples over P_2O_5 may be the most suitable technique as it avoids both the possible damage caused by high temperatures and the unknown effects of freeze-drying on the mechanical properties of hoof horn. The results from this drying technique showed a normal distribution together with a low variability, indicating that this may be an efficient technique for the dehydration of hoof horn.

As there were significant differences between the median moisture content results from many of the techniques, it was decided to determine moisture contents by dehydration over P_2O_5 for five days as the standard approach for future assessment of moisture content in donkey hoof horn. This technique would then avoid the possible effects of heat or cold on the protein structure and would also avoid possible subsequent alterations of the hoof horn prior to mechanical testing.

A standardised technique would also avoid the problems of looking at the effects of moisture content on the mechanical properties of hoof horn when different techniques have been used to assess moisture contents. For this reason comparisons of mechanical testing results have proved difficult in the past.

Some advantages and disadvantages of the different drying methods are summarised in Table 2.6.

Table 2.6 - Advantages and Disadvantages of the Drying Methods

Drying Technique	Advantages	Disadvantages
Phosphorus Pentoxide	Avoids sample heating	Drying time - 5 days
Freeze-drying	The sample should remain with an open porous structure, facilitating reabsorption of water (Nonhebel and Moss 1971). Avoids loss of another component which would have evaporated with the water at a higher temperature (Nonhebel and Moss 1971). Avoids denaturation of proteins (Mellor 1978). Drying time - 3 days.	Unknown effect on mechanical properties.
Air Drying at Room Temperature	Avoids sample heating	Drying time - 5 days. Not controlled for temperature, relative humidity or air movement. Cannot be used for samples that absorb moisture strongly.
Vacuum Drying (at Room Temperature)	Avoids sample heating.	Drying time - 3 days. Air entering is not controlled for relative humidity. Possible decrease in starch content (Lindroth and Koss 1996)
Oven Drying - Various Temperatures	Drying time - 24 hours	Volatilisation/decomposition (Willits 1951). Possible splitting of sample if moisture gradient is too steep (Pratt 1986).

2.5.2.6 The Effect of Age, Gender and Pigment on the Moisture Content of Donkey Hoof Horn

There was no significant difference in hoof moisture content between males and females which is contrary to the findings of Miyaki *et al* (1974) for horse hoof who found that the moisture content from the hooves of females was lower than that of males. Samples were dried at 107.5°C. There was also no significant difference between the moisture content of pigmented and *non*-pigmented hoof horn which is in agreement with previous authors (Benedetti 1948; Miyaki *et al* 1974; Leach 1980; Naumann 1984; Ley *et al* 1998). No seasonal effect on moisture content would have been expected as samples were taken at similar times. There was also no effect of age on the moisture content of donkey hoof horn. The animals were all housed under similar conditions so any environmental differences would not have been seen.

2.5.3 Comparison of the Moisture Content of Horse Hoof Horn with Those Results from Previous Authors for Horse and Pony Hoof Horn

The moisture content results of 26% for horse hoof horn from this study for samples dried over P_2O_5 compares well to the results of 25%, 27% and 27% found respectively by Sassen (1938), Miyaki *et al* (1974) and Butler (1976) and is just below the 28% found by Spitzlei (1998). However, the moisture content result for horse hoof horn found by Benedetti (1948) was 36%. This high result may have been due to the use of morbid samples and the different drying regime. Ley *et al* (1998) also found higher values of between 31-34%. They did, however, include hoof from the heel area which may be a possible reason for their results being higher.

On analysis of the data for pony hoof horn included in Reilly (1999) for moisture content for both treatment and control groups (n=4 in both groups), there was no significant difference between the moisture contents for both groups ($p>0.05$). The data were therefore combined, resulting in a mean moisture content of 23% (SD 5) which is lower than that found in the present study. Samples were, however, taken from morbid hooves which may have influenced the results. There was also a large range of 13-29% for these particular samples which may have been due to poor environmental control during the drying of samples at room temperature. A direct comparison was made between the data for pony hoof horn from Reilly (1999) and the data for horse hoof horn from the present study. There was no significant difference between the two data sets ($p>0.05$, Student's *t* test), indicating that there is no difference between the moisture contents of pony and horse hoof horn.

There was also no significant difference between the moisture regain of 36% for horse hoof from this present study and that shown for pony hoof horn of 30% (Reilly 1999) ($p>0.05$, Student's *t* test).

2.5.4 Comparison of the Moisture Content and Moisture Regain of Donkey and Horse Hoof Horn in This Study

Section 2.5.2.1 encompassed comparisons of the moisture content of donkey hoof horn with those found previously for horse and pony hoof horn. Direct comparisons proved difficult owing to different methodologies and drying techniques used by other authors. This section therefore presents a direct comparison of the moisture content of donkey and horse hoof horn that was analysed in this present study using similar collection, storage, preparation and analysis techniques.

Both the moisture content and moisture regain of donkey hoof horn were significantly higher than for horse hoof horn. This indicates a difference between the moisture content and moisture regain for the two species. This is also contrary to that shown in section 2.5.2.3 for median moisture content between pony and donkey hoof horn when there was no significant difference between the median moisture contents. It should be borne in mind that these latter results were for pony hoof horn and were not for horse hoof horn. As the pony study (Reilly 1999) was carried out under controlled trial conditions, it could be argued that these results therefore only apply to those particular conditions.

The difference indicated in this present study between the moisture content of donkey and horse hoof horn could possibly be due to differences in tubular morphology, protein or glycosaminoglycans (GAGs) content. Indeed, GAGs play an important role in regulating the amount of water in connective tissue (Junquiera 1971). GAGs are extremely hydrophilic and therefore combine with a great number of water molecules. Rothman (1954, cited in Yates 1970) showed that approximately 50% of the water in skin is associated with proteins and GAGs. Further investigations need to examine the role of these factors on the moisture content of hoof horn. Another question to be investigated that was raised in the literature review was whether or not the matrix constitutes the major water holding capacity of hoof horn. If the matrix of donkey and horse hoof horn is different, then

this may result in the differences in the moisture content of hoof horn between the two species.

As the moisture content of donkey hoof horn has been shown to be considerably greater than horse hoof horn in this study, it is proposed that an investigation is carried out on hoof horn from donkeys and horses kept on the same farm. These would need to be kept under similar management regimes as the different environments may have influenced the moisture content results. The moisture content of donkey hoof horn obtained from their indigenous countries would provide information as to whether or not the moisture content of donkey hoof horn in this country is, indeed, too high and management regimes should then be altered accordingly. It appears that donkeys in the UK are particularly susceptible to white line disease and seedy toe (Hopegood, L. personal observations). This susceptibility may be due to the high moisture content providing an ideal environment for multiplication of the bacteria and fungi that are implicated in these diseases. An alteration in the management of the donkeys may therefore reduce the susceptibility of these animals to these particular hoof problems.

2.5.5 Future Work

It is evident that there is still a great deal of experimental work to be carried out in the determination of moisture content within different areas of the hoof wall, together with comparative work between donkeys and other species such as cattle. A comparative study would increase the knowledge base and would allow further examination of the influence of moisture content on the structure and function of hoof horn.

Ideally samples should also be collected from animals kept under different regimes and in different geographical areas to ascertain any environmental effects on hoof moisture content. Sample sites from the whole hoof capsule should also be examined to see if there is, indeed, a proximo-distal moisture gradient. The standardised protocol for the analysis of moisture content may also be used to

question whether pathological conditions affect the moisture content of donkey hoof horn.

The use of more sophisticated approaches in the study of the moisture content of donkey hoof horn may provide an insight into where water molecules are sited within samples. This may reveal reasons as to how water acts as a plasticiser in hoof horn. Examples of advanced techniques that could be included are electron paramagnetic resonance spectroscopy, nuclear magnetic resonance spectroscopy, infra-red spectroscopy, impedence techniques and dielectric spectroscopy. Differential scanning calorimetry (DSC) could be used to measure the heat exchanges as the temperature of the sample is raised at a constant rate. This may indicate how the water is bound within hoof horn.

In skin, glycosaminoglycans bind together cells, providing toughness and flexibility and act as a cementing material. They also control electrolytes and moisture content (Elden 1971). The quantification of glycosaminoglycans within hoof horn may also be useful in studying its moisture content.

The effects of freeze-drying on the mechanical properties of hoof horn should be ascertained. The ability of hoof horn to reabsorb moisture following freeze-drying should also be examined.

An "optimal" level of donkey hoof horn hydration is yet to be discovered. Continuation of research into this field may provide the answers to ensure that management procedures enable the functions of the hoof to be achieved.

2.6 Conclusions

- Samples for moisture content analyses must be protected against moisture loss immediately following removal from the animal. Until there is more evidence to suggest otherwise, morbid hoof horn samples should also be taken and stored as quickly as possible after death to avoid possible changes in hoof moisture content;

- Different amounts of water can be removed from samples by different drying techniques. Standard sampling, storage and drying procedures must therefore be adopted to provide accurate moisture determination;

- There was a significant positive correlation between oven temperature and median moisture contents of donkey hoof horn;

- It is proposed that the moisture content of donkey hoof horn is assessed following drying of samples over phosphorus pentoxide for five days. Objective comparisons can then be made when examining the moisture content of hoof horn or when examining the influence of moisture on the mechanical properties of hoof horn;

- It is proposed that moisture content should be calculated as a percentage of fresh or original mass;

- The mean moisture content of donkey hoof horn, assessed following the drying of hoof horn over phosphorus pentoxide, was 33% and the moisture regain was 50%;

- There was no significant difference in the moisture content of hoof horn samples taken from males or females;

- There was no significant difference in the moisture content of samples from pigmented and *non*-pigmented hoof horn;

- There was no relationship between animal age and the moisture content of donkey hoof horn;

- The mean moisture content of horse hoof horn was 26% and the moisture regain was 36%;

- There was a significant difference between the moisture content and moisture regain of donkey and horse hoof horn. This indicates a difference between the two species.

3. ZONAL MOISTURE CONTENT OF THE *STRATUM MEDIUM* OF DONKEY HOOF HORN

3.1 Introduction

As has been discussed in section 1.8 of the literature review, the moisture content of hoof horn affects its function, quality and mechanical properties. It is believed, for horse hoof, that a dorso-palmar moisture gradient exists across the hoof wall depth (Leach 1980; Douglas *et al* 1996) (section 1.8.2.4.2). However, these authors only divided the hoof wall into inner and outer wall samples. Whether a similar gradient exists for donkey hoof horn is not known. The existence of such a dorso-palmar moisture gradient may have a profound effect on the mechanical properties of the hoof horn.

The determination of the moisture content at various sites across the HWD would test the existence of a dorso-palmar moisture gradient for donkey hoof horn. The results would then enable future mechanical testing of partial HWD samples to be carried out at a level of moisture content that would be equivalent to an *in vivo* moisture content. When the moisture content across the HWD is known, the moisture content of differing regions can be manipulated *in vitro* to the same level for each region. This would then provide an indication as to whether moisture content alone is responsible for the difference in the mechanical properties of hoof horn.

As mentioned in section 1.4 of the literature review, the study of Reilly *et al* (1996) for pony hoof and Reilly *et al* (1998b) for horse hoof, have shown that there is a dorso-palmar decrease in tubule density across the HWD, thus providing a four-zoned structure. Tubule density was examined in this present thesis for donkey hoof horn and is reported in Chapter 5. Other authors have chosen to mechanically test samples that cover only part of the *Stratum medium* (for example, Leach 1980; Leach and Zoerb 1983; Douglas *et al* 1996). Their specific sample areas will be discussed in Chapter 6. More recently, Kasapi and Gosline (1999) suggested that the mechanical properties of hoof vary through the HWD. If both these important characteristics of tubule density

and mechanical properties appear to change in a dorso-palmar direction across the HWD, it is possible that moisture content will also alter across the HWD for donkey hoof horn. An increase in the knowledge base about the existence or not of a dorso-palmar moisture gradient would further contribute towards the understanding of how moisture content affects the function of the hoof.

Although the tubule density of pony and horse hoof is shown to exist in zones (Reilly *et al* 1996, 1998b), it is unlikely that the moisture content of hoof horn follows such a stepped pattern unless tubule density has a very great influence on the moisture content of hoof horn. If the moisture content changes at all across the HWD it is likely to change in a continuous dorso-palmar gradient as a result of the effect of osmosis rather than in a stepped pattern. A continuous change in moisture content would be difficult to assess. The present project therefore examined the moisture content in four zones across the HWD of donkey hoof horn.

As the moisture content for horse hoof horn has not been reported in the literature for four zones, horse hoof horn was examined for comparative purposes in this study.

3.2 Aim

The aims of this part of the study were to:

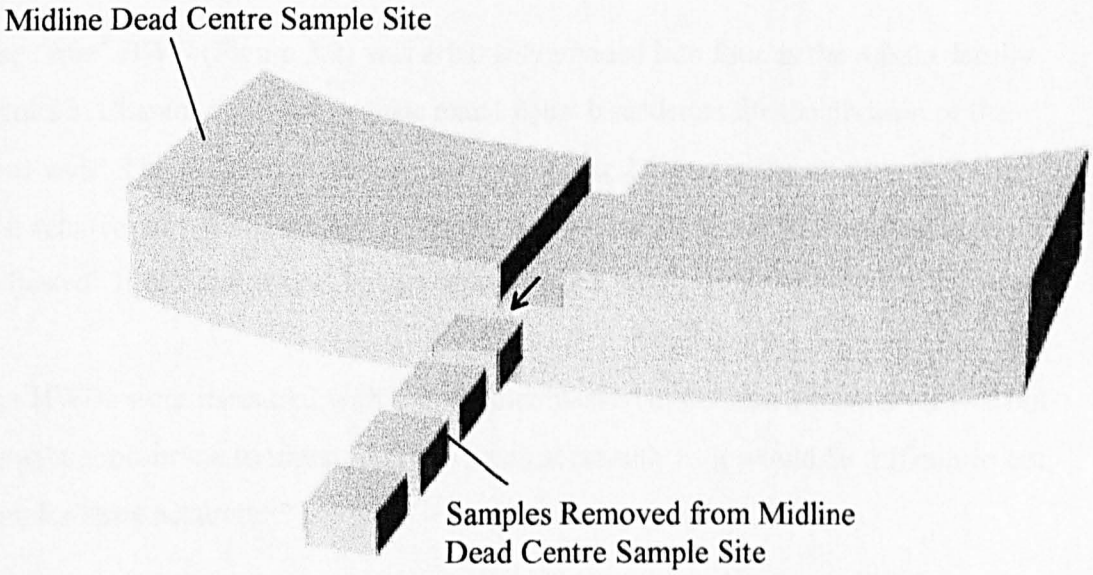
- establish the moisture content across the *Stratum medium* of donkey hoof horn and thereby ascertain the existence, or not, of a dorso-palmar increase in moisture content;

- establish the moisture content across the *Stratum medium* of horse hoof horn and compare the results to those for donkey hoof horn.

3.3.1 Donkey Hoof Clippings

Hoof clippings were obtained from the left fore limb of ten donkeys which are identified in Appendix 1. One sample of 1.5 mm x 1.5 mm by HWD was removed from each MDC section. An example of a sample removed from a MDC section and divided into four is shown in Figure 3.1.

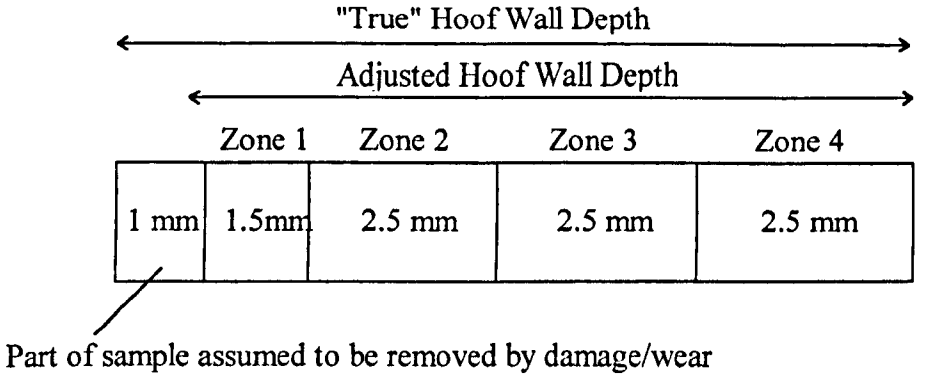
Figure 3.1 - An Example of a Sample Removed from a Midline Dead Centre Section and Divided into Zonal Moisture Sections (divisions not to scale)



As preliminary work in Chapter 5 had indicated that the outermost 10% of the clipping was missing, the HWDs were adjusted accordingly to take this into account. As an example, a clipping with a 9 mm HWD was likely to have been equivalent to

90% of the original HWD. The original clipping size would therefore have been 10 mm (Figure 3.2).

Figure 3.2 - 10 mm Sample of Donkey Hoof Clipping Taking into Account Missing 10% Hoof Wall Depth (not to scale)



The "true" HWD (Figure 3.2) was arbitrarily divided into four as the tubule density results in Chapter 5 did not provide exact zonal boundaries for the division of the hoof wall. This resulted in each zone representing 2.5 mm in the example provided. The removal of 10% of the HWD, that is 1 mm for a 10 mm HWD resulted in an "adjusted" HWD and zone 1 being reduced to 1.5 mm.

The HWDs were measured with a steel ruler marked at 0.5 mm intervals. It was not thought appropriate to measure HWD more accurately as it would be difficult to cut samples more accurately.

An example of the calculation used to assess sizes of zonal sections for a 9 mm HWD is provided.

A HWD of 9 mm represents 90% of the HWD. To calculate the original 100% HWD:

100/9 = 1.1 x 9 mm = 10 mm = "true" HWD

This was then divided into four to provide zones at 2.5 mm intervals. The measurements for zones 2, 3 and 4 were added together to result in a total of 7.5 mm. This was then subtracted from the HWD of 9 mm to give a zone 1 measurement of 1.5 mm. Samples were cut using a scalpel as close to the expected zonal divisions as possible.

The moisture contents for each zone were assessed following drying of the samples over phosphorus pentoxide using the method given in section 2.5.2. Again, moisture contents were calculated as a percentage of fresh mass.

3.3.2 Horse Hoof Clippings

Samples were removed from the MDC of the sixteen horse hoof clippings that were used in section 2.4.3 for full HWD analysis of moisture content. The preparation was the same as for donkey hoof clippings. As an estimation could not be made of the amount of wear these samples had incurred, the samples were divided into four equal sections.

3.4 **Results**

3.4.1 Hoof Wall Depths

The results for "true" HWD and zonal measurements for donkey hoof horn are shown in Table 3.1.

Table 3.1 - Results for "True" Hoof Wall Depth and Zonal Measurements of Donkey Hoof Horn

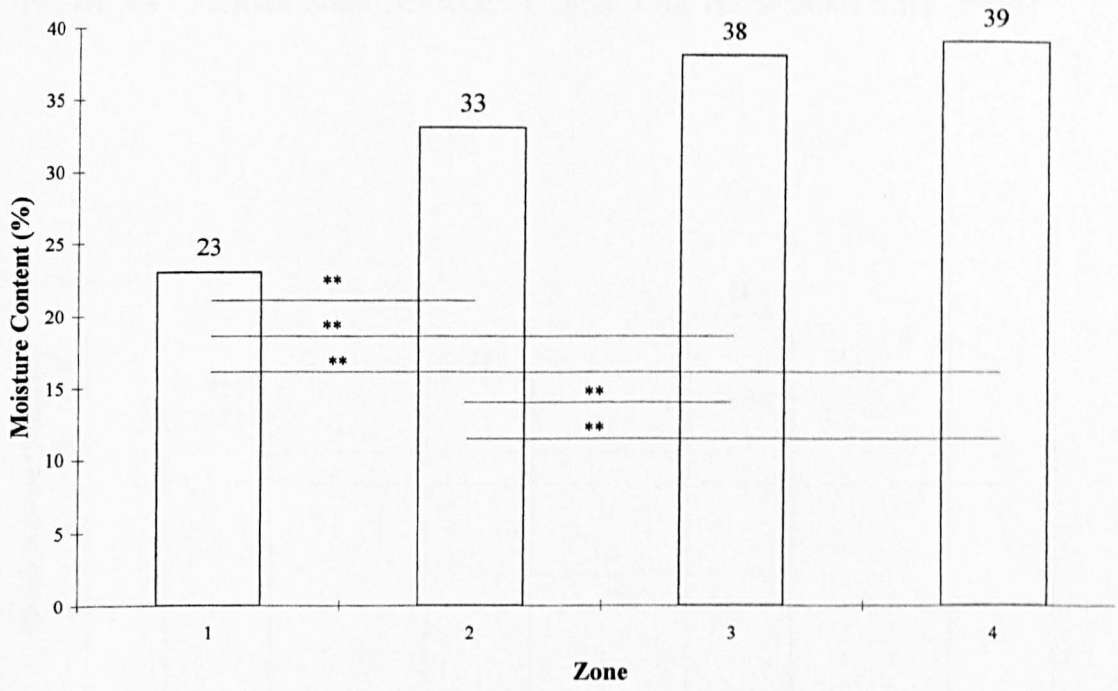
Original HWD (mm)	"True" HWD (mm)	Zonal Widths (Zones 2, 3 and 4) for 100% HWD (mm)	Zone 1 (mm)
9.0	10.0	2.5	1.5
7.0	7.8	1.9	1.2
7.0	7.8	1.9	1.2
8.0	8.9	2.2	1.3
7.0	7.8	1.9	1.2
8.0	8.9	2.2	1.3
8.0	8.9	2.2	1.3
8.0	8.9	2.2	1.3
10.0	11.1	2.8	1.7
7.0	7.8	1.9	1.2

3.4.2 Donkey Hoof Horn

The zonal moisture content results for donkey hoof horn are shown in Figure 3.3. The zonal moisture contents were not normally distributed ($p<0.05$) and therefore median values were used to describe the data set. There was a median moisture content of 35% for the full data set (Appendix 5).

There was a dorso-palmar increase in median hoof moisture content. There were significant differences between moisture contents for individual zones ($p<0.01$, Kruskal-Wallis) (Figure 3.3). Mann-Whitney *U* tests indicated there were significant differences between the combination of all zones ($p<0.01$) except for between zones 3 and 4 ($p>0.05$).

Figure 3.3 - Median Zonal Moisture Contents for Donkey Hoof Horn (n=10)



Key: —**— denotes signifcant differences between zones at $p<0.01$

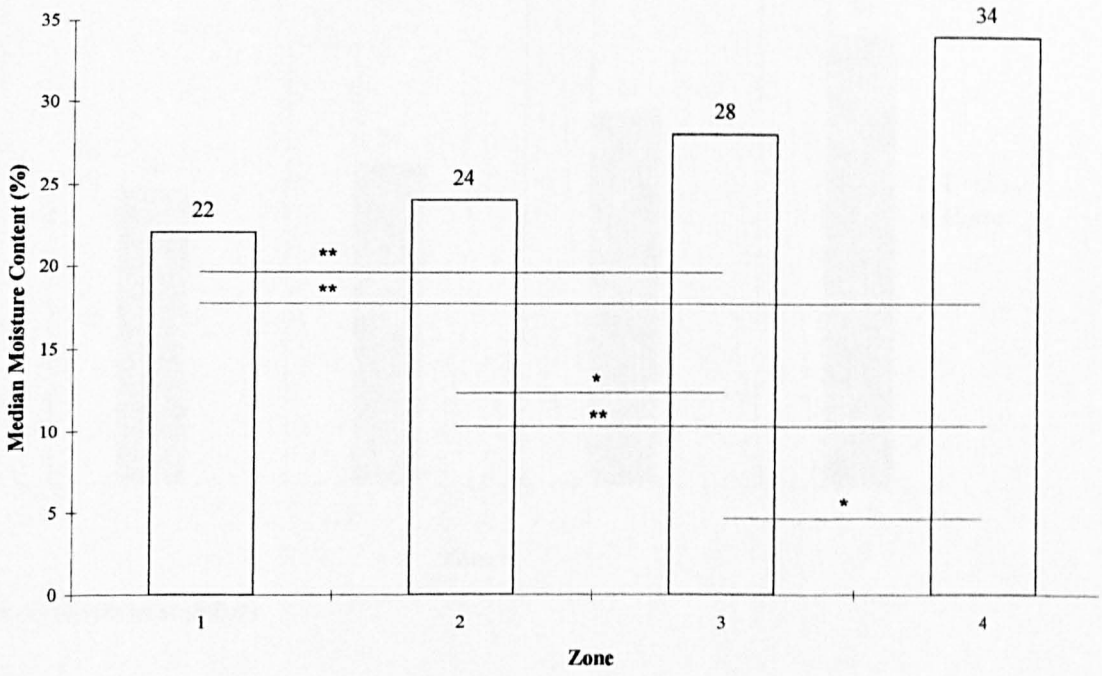
3.4.3 Horse Hoof Horn

The zonal moisture content results for horse hoof horn are shown in Table 3.2 and Figure 3.4 with the full data set in Appendix 6. The zonal moisture contents for zones 1-3 were normally distributed but for zone 4 was *non*-normally distributed. There was a dorso-palmar increase in median hoof moisture content. There was no significant difference between zones 1 and 2 ($p>0.05$, Mann-Whitney U test). There were, however, significant differences between all other combinations of zones ($p<0.05$, Mann-Whitney U test).

Table 3.2 - Zonal Moisture Contents for Horse Hoof Horn

Zone	Mean (%)	Median (%)	Standard Deviation	Coefficient of Variation (%)	*n*
1	22	22	6	27	16
2	24	24	4	17	16
3	27	28	4	15	16
4	N/A	34	N/A	N/A	16
Full Data Set	N/A	25	N/A	N/A	64

Figure 3.4 - Median Zonal Moisture Contents for Horse Hoof Horn (n=16)

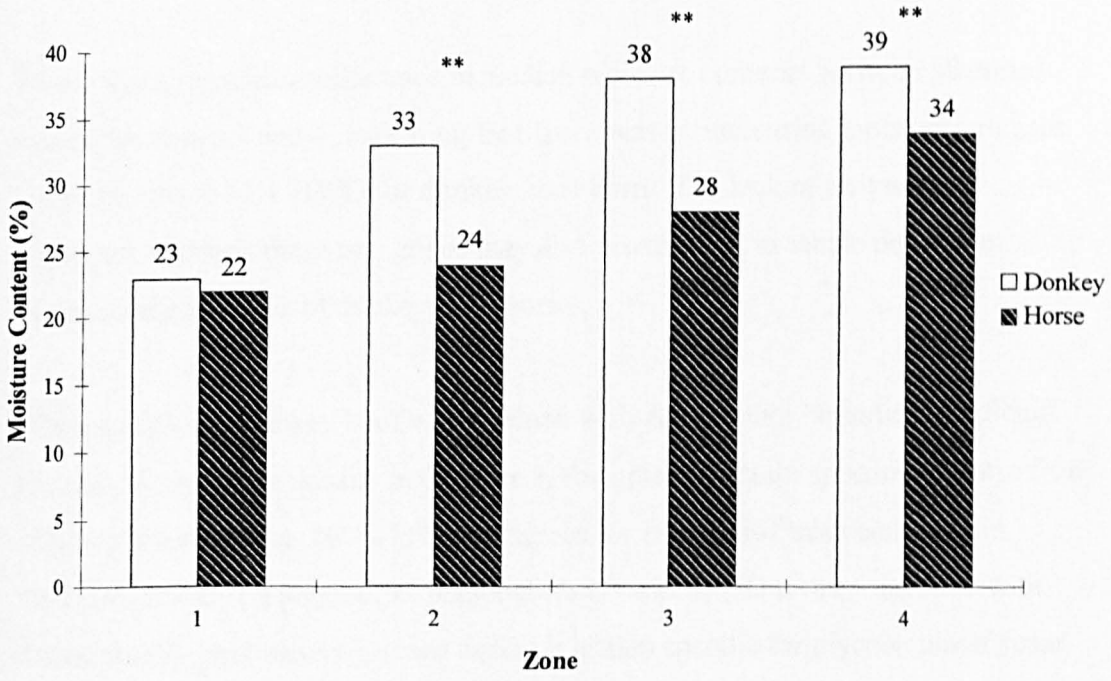


Key: * denotes signifcant differences between zones at $p<0.05$
** denotes significant differences between zones at $p<0.01$

3.4.4 Comparison of Moisture Content for Donkey and Horse Hoof Horn

A comparison of moisture contents for donkey and horse hoof horn is shown in Figure 3.5. There was no significant difference between the median moisture content of zone 1 for both donkey and horse hoof horn ($p>0.05$, Mann-Whitney *U* test). However, there was a significant difference between the median moisture contents of zone 2, zone 3 and zone 4 for both donkey and horse hoof horn ($p<0.01$, Mann-Whitney *U* test).

Figure 3.5 - Comparison of Median Zonal Moisture Contents for Donkey and Horse Hoof Horn



** denotes significance at $p<0.01$

3.5 Discussion

3.5.1 Donkey Hoof Horn

The examination of the full HWD does not take into account possible differences in moisture content across the HWD which have been investigated by dividing the full HWD into zones. The results obtained from donkey hoof horn indicated that there was, indeed, a dorso-palmar increase in moisture content across the *Stratum medium* of donkey hoof horn. Although the analysis of moisture content across the HWD has been examined according to zones, this does not infer that the moisture content of the *Stratum medium* of donkey hoof horn does not change very gradually across the HWD. The use of zones provides a median value of moisture content across the individual zones.

The greater moisture content of the inner wall of donkey hoof samples when compared to outer wall samples parallels the quantitative findings of Leach (1980) and Douglas *et al* (1996) for horse hoof.

There was a significant difference in median moisture contents between all zones except for zones 3 and 4, indicating that there was no increasing moisture gradient across the inner 50% HWD for donkey hoof horn. The lack of a significant difference between these two zones may also be mirrored in tubule density or mechanical properties of donkey hoof horn.

When samples of donkey hoof were stained with Alcian Blue Periodic Acid Schiff for analysis of tubule density in Chapter 5, the uptake of stain appeared greater from approximately 40% to 100% HWD, whereas for horse hoof this occurrs from 70-100% HWD (Hopegood, L. personal observations). This may correlate with differences in glycosaminoglycans as this is a stain specific for glycosaminoglycans (Wheater 1987). Kasapi and Gosline (1999) believed that the moisture content of hoof horn was also associated with the protein content but did not expand further on this idea.

It was thought that the distance of samples from the hydrating fluids of the dermis is an important factor for hoof moisture content (Leach 1980). However, for donkey hoof horn, the similarity between zone 3 and 4 may indicate that a factor other than distance from the dermis is involved in the uptake of water in this area, such as the chemical composition. For example, there may be a high level of glycosaminoglycans present which would influence the water binding properties of the *Stratum medium*. The 38% moisture content for zone 3 and the 39% moisture content for zone 4 may indicate an optimal hydration for the function of these parts of the wall.

Douglas *et al* (1996) believed that the gradient of stiffness brought about by the difference in moisture content between the outer horn and soft tissues of the dermis would help reduce stress values at the interface between the epidermis and dermis

and would reduce the magnitude of the peak stress experienced by the soft tissues during locomotion. Kasapi and Gosline (1999) also believed that the gradual change in stiffness across the wall, partly arising as a result of the proximity of the tissue to a source of moisture (Leach 1980) provided for a more gentle transfer of loads to the collagenous suspensory elements of the dermis. The more upright hoof in the donkey than in the horse (Reilly 1997) may cause greater stress in the donkey hoof wall compared to the horse. This problem may be counteracted by the mechanism of the higher moisture content in the inner hoof wall of the donkey. Indeed, Dorrington (1980) believed that the amount of water contained in a biological material may be a function of the stresses applied to it, resulting in the ability to increase or decrease tissue volume as necessary.

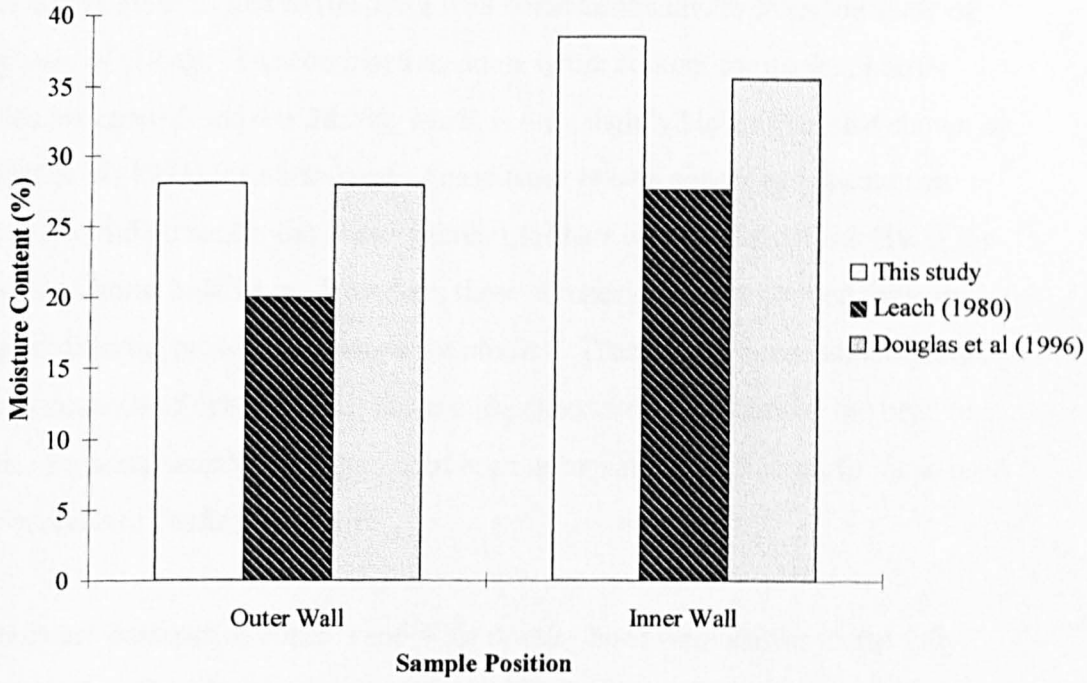
Hunting (1899) believed that a hoof in dry weather becomes hard and will withstand wear on the hardest of roads. The lower moisture content in the outer wall of donkey hoof may therefore be one way in which the hoof copes with the influence of the external environment. Indeed, Naumann (1984) found an increase in the wear of horse hoof containing a high water content. A wet environment may therefore increase wear on the hoof whereas with a dry environment wear would be reduced.

3.5.2 Comparison of the Zonal Moisture Content of Donkey Hoof Horn with Results Previously Found in the Literature on Horse Hoof Horn

The zonal moisture contents for zones 1 and 2 for donkey hoof horn shown in this study were averaged to provide an "outer" hoof wall comparison. The zonal moisture contents for zones 3 and 4 were also averaged to provide an "inner" hoof wall comparison. Again, it must be acknowledged that comparisons with results from previous authors are difficult owing to different protocols.

A comparison of results for donkey hoof horn with those from previous authors for horse hoof horn is shown in Figure 3.6. Although the results of Leach (1980) are lower for both outer and inner wall than for those in this study, the results from Douglas *et al* (1996) are, in fact, higher than those presently seen. These differences are discussed in detail.

Figure 3.6 - Comparison of the Moisture Content of "Outer" and "Inner" Donkey Hoof Wall Samples with those from Previous Authors for Horse Hoof Horn



Leach (1980) found that outer wall samples of horse hoof possessed significantly less water within a range of 18.5-21.8% than inner wall samples with the average moisture content of outer wall samples being 20% and inner wall samples being 27.6% (Table 1.5) with a range of 22.7-30.5%. These results indicate a dorso-palmar increase in moisture content. The slight differences between the present results and those of Leach (1980) may be explained as morbid samples were included in his analysis and were dried at 60°C under vacuum. The divisions of the zonal boundaries were also not identified.

Douglas *et al* (1996) established the moisture content of 'dorso-proximal' samples which were approximately a third of the distance down the hoof wall, and 'dorsal-distal' samples which were approximately two thirds of the distance down the hoof wall. The full wall thickness was then divided into two to produce outer and inner wall samples. Samples were dried to a constant mass at 103.5°C. Inner wall samples

had a significantly higher moisture content of 35.5% when compared to those of the outer wall samples of 27.9%. If the present results for donkey hoof clippings from zones 1 and 2 are combined and the mean calculated, this produces a value of 27.5% which is very close to that of the outer wall horse hoof samples from the study of Douglas *et al* (1996). The combined mean moisture content for donkey hoof samples for zones 3 and 4 is 38.5%, which is only slightly higher than that shown by Douglas *et al* (1996) for horse hoof. These latter results appear to indicate that there are no differences in the dorso-palmar moisture gradient across the HWD for donkey and horse hoof horn. However, these comparisons have proved difficult owing to differing protocols between the studies. These differences include sample site and methods of dehydration. These comparisons also emphasised the need to examine separate samples for horse hoof horn using similar techniques to those used in the analysis of donkey hoof horn.

The moisture contents of zones 3 and 4 for donkey hoof were similar to the fully hydrated full wall thickness samples produced by Bertram and Gosline (1987) which resulted in a moisture content of 40.2%. However, it should be borne in mind that their samples were dried at 80°C for five days. It would be expected that if their samples had been dried by placing them over phosphorus pentoxide their moisture content would have increased to at least 45% as the difference between samples dried at 90°C and those over phosphorus pentoxide in this present study showed a difference of 5%. This value of 45% would be greater than that shown for donkey hoof or, indeed, shown by Douglas *et al* (1996) for horse hoof.

Bertram and Gosline (1987) carried out fracture tests *in vitro* on fully hydrated horn and found that it is more prone to crack propagation than normally hydrated horn. Thomason *et al* (1992) believed that the main effect of the dorso-palmar moisture gradient was to provide a high level of fracture toughness. The lower moisture content in the outer wall may therefore provide protection to the inner wall and sensitive structures. If an excess of moisture existed in this protective layer then this may lead to cracks and possible catastrophic failure of the hoof wall.

The differences in moisture content across the hoof wall may be partly due to tubule morphometry as Kasapi and Gosline (1999) had previously found, for horse hoof, that the medullae of tubules from the mid wall occupy 4.5% of the hoof wall area whereas those from the inner wall only occupy 1.5%. There is therefore less *Stratum medium* available in the outer wall to absorb water, therefore resulting in a lower water content. If the sizes of the medullae for donkey hoof are similar to those for horse hoof, this would probably only change the moisture content by 1% or 2% which would not account for the differences in zonal moisture content in these areas.

As mentioned earlier, the uptake of water in keratins by the matrix is thought to be considerably higher than the uptake by the IFs (Fraser *et al* 1971). Kasapi and Gosline (1999) found, for horse hoof, that there was no significant difference between the IF volume fraction for inner wall intertubular horn and that of the inner wall tubular horn. However, these IF volume fractions for intertubular horn for outer wall and mid wall were 30% and 35% greater than for the inner wall. They then found, however, that these differences in volume fraction did not correlate well with the fully hydrated moisture contents for the inner, mid and outer wall of 48%, 41% and 35% respectively found by Kasapi and Gosline (1997). They believed that, according to volume fractions, the mid and outer walls should have had the same moisture content. If these IF volume fraction differences occur in donkey hoof, the lower inner wall IF volume fraction, at least in part, may contribute to the higher moisture content found here for zones 3 and 4 as more matrix would be available for the uptake of water. However, Kasapi and Gosline (1999) believed that differences may be due to protein type and content changing across the hoof wall depth.

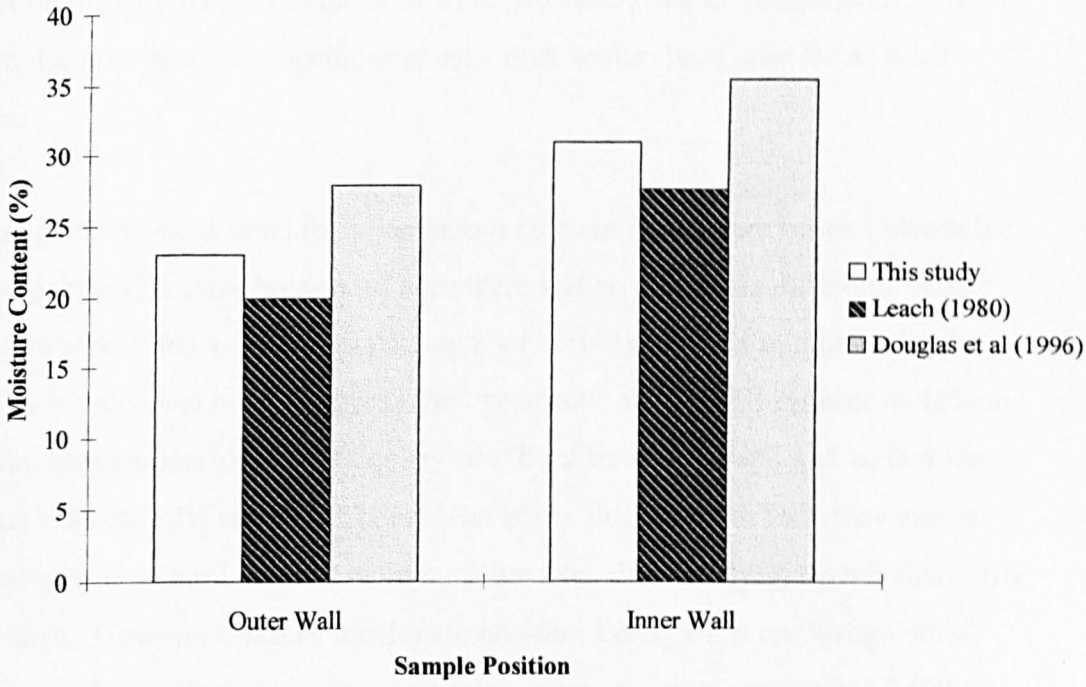
3.5.3 Comparison of the Zonal Moisture Contents of Horse Hoof Horn in the Present Study with that Previously Found for Horse Hoof Horn

The zonal moisture contents for zones 1 and 2 for horse hoof horn shown in this study were averaged to provide an "outer" hoof wall comparison. The zonal moisture contents for zones 3 and 4 were also averaged to provide an "inner" hoof

wall comparison. Again, it must be acknowledged that comparisons with results from previous authors are difficult owing to different protocols.

A comparison of results for horse hoof horn with those from previous authors is shown in Figure 3.7.

Figure 3.7 - Comparison of the Moisture Content of "Outer" and "Inner" Horse Hoof Wall Samples with those from Previous Authors for Horse Hoof Horn



Although the moisture content results of Leach (1980) are lower for both outer and inner wall samples than for donkey hoof horn, those results from Douglas *et al* (1996) are, in fact, higher than the moisture content results for donkey hoof horn. These differences may be due to the differing sample sites and protocols that were identified in section 3.5.2.

3.5.4 Comparison of the Zonal Moisture Contents of Donkey and Horse Hoof Horn from this Study

As zonal moisture contents have not been reported previously for donkey hoof horn, a direct comparison with other work could not be made. As mentioned in section 3.5.2, comparisons with other work have proved difficult. Examination of the zonal moisture content for horse hoof horn was therefore carried out in this study for comparative purposes only.

There was no significant difference between the moisture content for zone 1 for both donkey and horse hoof horn. This may be because the environment has a similar effect on the external part of the hoof wall. However, further comparisons showed that horse hoof horn was significantly drier than donkey hoof horn for all other zones.

Although there was a trend for a continuous increase in moisture content across the dorso-palmar HWD for horse hoof horn there was no significant difference shown between zone 1 and zone 2. For donkey hoof horn there was a significant increase of 10% in moisture content between the two zones. The overall increase of 16% in the moisture content shown for donkey hoof horn between zone 1 and zone 4 was greater than the 12% shown for horse hoof horn. Both of these facts may indicate that donkey hoof horn receives more moisture from the underlying dermis than horse hoof horn. However, reasons for this are unclear. Perhaps it is a self-regulating mechanism by which the possible concussive effect of a more upright hoof of the donkey is counteracted by the actual moisture as this factor is known to influence the mechanical properties of hoof horn (Chapter 6).

As well as significant differences between the moisture content of donkey and horse hoof horn for full HWD samples, significant differences are now known to exist between zones 2, 3 and 4 for donkey and horse hoof horn. However, it must be borne in mind that the zonal boundaries were slightly different between the two data sets as the wear of the samples of horse hoof had not been taken into account.

3.5.5 Future Work

Various questions still need to be addressed. For example, as to whether, for donkey hoof horn, tubule morphometry, IFs, protein and GAGs content are, in part, responsible for the differences in moisture content of hoof horn across the *Stratum medium*. Quantitative analyses of these parameters in conjunction with assessment of moisture content of donkey hoof horn would enable these questions to be answered. Further examination of the moisture content around the remainder of the clipping and the rest of the hoof capsule to provide a "water map" is needed. This could then be related to the function of the hoof.

Moisture loss or gain from the outer hoof wall should be examined, together with the contribution of moisture content from the underlying dermis.

3.6 Conclusions

- There was an increase in dorso-palmar moisture content across the *Stratum medium* of both donkey and horse hoof horn;

- There was no significant difference between the moisture content for zone 3 and zone 4 for donkey hoof horn;

- There was no significant difference between the moisture content for zone 1 and zone 2 for horse hoof horn;

- There were significant differences between zones 2, 3 and 4 between donkey and horse hoof horn;

- A steeper dorso-palmar moisture gradient was shown for donkey hoof horn when compared to horse hoof horn;

- Zonal moisture content analysis demonstrated subtle differences in moisture content across the hoof wall that were not seen when the full hoof wall depth was examined.

4. ALTERNATIVE METHODS OF HYDRATING HOOF HORN

4.1 Introduction

As has been identified in Chapter 2, it is not always possible to maintain hoof horn moisture content during collection, storage and preparation of samples prior to examination. The utilisation of fully hydrated samples to achieve hydrated moisture content provides a consistent level of hydration for sample analyses, for example, prior to mechanical testing. The hydrated moisture content has not been reported for donkey hoof horn.

Again, methods of carrying out the hydration process, together with methods of calculation of hydrated moisture content have not been explained or have not been consistent. Many authors using hydrated samples have not explained their protocols fully (Benedetti 1948; Bertram 1984; Naumann 1984; Spitzlei 1996).

4.1.1 Methods of Calculating Hydrated Moisture Content

The following methods of calculating HMC each have their own advantages or disadvantages.

The hydrated moisture content calculation (Equation 10) is based on a percentage of hydrated mass, the result cannot be compared to other moisture contents calculated as a percentage of dry or *in vivo* mass.

Equation 10 Hydrated moisture content $= \frac{WW - DW}{WW} \times 100$

Key for Equation 10 and Equation 11

WW	Wet weight	Mass of sample following hydration
DW	Dry weight	Mass of sample following no further mass loss after drying

Hydrated moisture content can also be calculated as a percentage of the dry mass. This indicates the mass of water that has been absorbed over and above the dry mass. In this study this is known as hydrated regain (HMC_D) (Equation 11). This method of calculation was used by Bertram and Gosline (1987) and Reilly (1999) where it was known as hydrated moisture regain. The hydrated regain can then be compared, as necessary, to actual moisture content calculated as a percentage of dry mass. Both the hydrated moisture content and hydrated regain are, in effect, "normalised" as they do not take into account an *in vivo* mass.

Equation 11 $$\text{Hydrated regain} = \frac{WW - DW}{DW} \times 100$$

It is clear that the method of determining hydrated moisture content needs to be presented as the calculations produce differing results. Use of HMCs provides a means of, firstly, normalising for moisture content prior to mechanical testing and, secondly, a means of ascertaining the level of saturation at which the hoof wall functions as this can be calculated from the raw data. This is believed to be between 70-90% for horse hoof (Zschokke 1885) but has not been reported for donkey hoof horn.

4.1.1.1 Drying Techniques Used in Assessing Hydrated Moisture Contents

Again, as for determining moisture contents, the drying techniques used to ascertain hydrated moisture contents vary (section 1.9.2.1). These include drying at 80°C for five days (Bertram and Gosline 1987), 90-100°C (Benedetti 1948), 100°C for five days (Kasapi and Gosline 1997) and 100-110°C (Naumann 1984; Spitzlei 1996). As with moisture content results, these different methods are likely to result in different values for hydrated moisture contents and will therefore make comparisons difficult. Placing samples over phosphorus pentoxide was identified in Chapter 2 as the standard protocol for drying samples of hoof horn. This method was then used to

assess hydrated moisture contents for both donkey and horse hoof horn as no data were available for a direct comparison of results with those from horse hoof horn.

4.1.2 Relative Humidity Environments

Another method of normalising the moisture content prior to further study is to place samples in controlled humidity chambers at specific levels of relative humidity until equilibrium mass is achieved.

Some authors have used this technique to hydrate horse hoof horn but reasons for its use are not clear (Bertram and Gosline 1987; Küng 1991; Geyer and Schulze 1994; Zenker *et al* 1995; Hinterhofer 1996; Hinterhofer *et al* 1998; Wagner *et al* 2001). The resultant moisture contents following equilibration have also not been provided (Geyer and Schulze 1994; Zenker *et al* 1995; Kasapi 1997; Kasapi and Gosline 1998; Wagner *et al* 2001). It has therefore been difficult to compare results from mechanical tests when sample moisture contents are unknown. Relative humidity environments have not been reported to have been used prior to subsequent analyses of donkey hoof horn.

Only four levels of relative humidity environment have been reported to have been used to produce a sorption isotherm for horse hoof (Bertram and Gosline 1987) and no information has been reported for either a sorption or desorption isotherm for donkey hoof horn. A greater range of environments should be employed to examine the sorption and desorption isotherms for donkey hoof horn as the use of four points cannot provide a representative sigmoidal shaped curve.

4.1.2.1 Relative Humidity Environments

Again, the drying techniques used to assess the hydrated moisture contents vary following equilibration in specific relative humidity environments. These include drying at 80°C for five days (Bertram and Gosline 1987), 100°C for five days (Kasapi and Gosline 1997), 105°C for forty eight hours (Küng 1991) and 110°C for fifty hours (Zenker *et al* 1995; Hinterhofer 1996; Hinterhofer *et al* 1998). The

different drying temperatures would result in different values for moisture contents as shown in Chapter 3.

In the experiments carried out by Bertram and Gosline (1987) samples from morbid hooves were equilibrated over phosphorous pentoxide to produce 0% RH. Other samples were equilibrated in 53% and 75% RH environments produced by placing the specimens over the relevant saturated salt solutions. The reasons for choosing these particular relative humidities were not explained. Samples were dried at 80°C for five days and moisture contents were calculated as hydrated regain. The hydrated regain results from the study of Bertram and Gosline (1987) for samples equilibrated at 0%, 53%, 75% and 100% RHs were 5.5%, 11.7%, 18.2% and 40.2% respectively.

Geyer and Schulze (1994) and Zenker *et al* (1995) stored samples at 65% RH and 20°C for ninety six hours. These authors, together with Kasapi (1997) and Kasapi and Gosline (1998) did not report the results for moisture contents following equilibration.

Hinterhofer (1996) and Hinterhofer *et al* (1998) conditioned horse hoof samples by storing them above a 65% RH environment at a temperature of 23°C for six days. The moisture content of the conditioned samples was 15.9%. This level of relative humidity had caused the sample to lose moisture to the surrounding environment. The authors had chosen this particular relative humidity in order to compare the work with that of Küng (1991) and Küng *et al* (1991) who also tested samples following storage for six days at 65% RH but at 20°C which produced samples with a slightly lower moisture content of 13.3%. Other factors such as different animals and differing sampling times may also possibly account for the differences.

The completion of a sorption and desorption isotherm may allow analysis of the hydration of donkey hoof horn and may identify a method of rehydrating samples to an *in vivo* moisture content. The use of equilibration of samples in these different

environments may achieve this aim. Again, the methods and drying techniques employed must be standardised.

When the "normal" hydrated moisture content for donkey hoof horn has been established, it may be that, if the hydrated moisture content is abnormally high, there may be a disease problem or a problem with loss of structure, for example, due to cracking of the hoof horn.

4.2 Aims

The aims of this part of the study were:

- to establish a method to normalise hoof moisture contents by using hydrated moisture contents;

- to assess hydrated moisture content and hydrated regain for full HWDs and for zones for the *Stratum medium* of donkey and horse hoof horn;

- to identify the level of saturation at which the hoof wall functions;

- to compare results for hydrated regain of donkey samples with those from pony samples from Reilly (1999);

- to investigate the hydration of donkey hoof horn by identifying the sorption and desorption isotherms and to see if these are similar to those previously described for other keratinous materials;

- to ascertain the relative humidity of an environment that may enable donkey hoof samples to be rehydrated to their *in vivo* moisture content;

- to provide a method of identifying moisture contents for horse hoof samples from the literature that had previously been equilibrated at specific relative humidities prior to mechanical testing.

4.3 Materials and Methods

4.3.1 Full Hoof Wall Depth Samples

A sample of approximate dimensions of 1.5 mm x 1.5 mm x full hoof wall depth was removed from the midline dead centre of each donkey hoof clipping from the left fore limb of five donkeys (Appendix 1). More samples were not available owing to hoof wear and some of the donkeys had developed white line disease and were not available to be used for analyses.

Samples were weighed to establish the *in vivo* mass and then dried over phosphorus pentoxide, as per the standardised method outlined in Chapter 2, and reweighed daily until attainment of equilibrium mass. The dried samples were then hydrated by placing in distilled water at room temperature. The samples were removed each day and the surface blotted lightly with absorbent paper to remove surplus liquid. The mass was then recorded for each sample. This procedure occurred daily until there was no further mass gain. Calculations were carried out to establish the hydrated moisture content (Equation 10) and hydrated regain (Equation 11).

The same sixteen horse hoof clipping samples described in Chapter 2 were used to assess hydrated regain for comparative purposes.

The percentage saturation of both donkey and horse hoof samples was found by calculating the fresh mass as a percentage of hydrated mass.

4.3.2 Zonal Assessment of Hydrated Moisture Contents

The ten samples used were the same as those in section 3.3 (Appendix 1). Following assessment of zonal moisture content, the above protocol was then repeated to establish hydrated moisture contents.

The same sixteen horse samples were used that were previously used to assess zonal moisture contents (Chapter 3). Again, only hydrated regain was used for comparative purposes. The percentage saturation of zonal samples was calculated according to section 4.3.1.

4.3.3 Production of Sorption and Desorption Isotherms for Donkey Hoof Horn

A sorption and desorption isotherm for donkey hoof samples only were produced by using saturated salt solutions to control relative humidity for both sorption and desorption experiments as this particular method had been used previously used by Bertram and Gosline (1987). Hoof clippings were obtained from the left fore limb of ten donkeys (Appendix 1). The MDC was divided into ten full HWD sections of 1.5 mm by 1.5 mm. Each sample was labelled 1-10 according to the position at the MDC. Sample 1 was the most lateral sample and sample 10 was the most medial sample. Samples 1-5 were used for sorption tests. Samples 6-10 were used for desorption tests. The relative humidity environments of saturated salt solutions used are outlined in Table 4.1. Phosphorus pentoxide was used to produce the environment at 0% RH. The remaining environments were established according to ASTM E104-85 (1996) and were kept at room temperature (23°C). The environments were tested using a relative humidity probe[4].

[4] Grant Instruments Ltd, 1001 series, Cambridge.

Table 4.1 - Salt Solutions Used to Produce Relative Humidity Environments

Media used to Produce Relative Humidity Environments	Humidity of the Environment (% Relative Humidity)
Phosphorus pentoxide	0
Lithium Chloride	11
Potassium Acetate	22
Magnesium Chloride	32
Potassium Carbonate	44
Potassium Dichromate	56
Sodium Nitrite	66
Sodium Chloride	76
Potassium Chloride	85
Potassium Nitrate	93

"Sorption samples" were weighed to ascertain their *in vivo* mass and were then dried over phosphorus pentoxide for ten days and reweighed to ascertain dry mass. One sample from each was then allocated to the different relative humidity environments and left for ten days in order for equilibrium to be achieved. Samples were then reweighed to ascertain hydrated mass. The hydrated regain was calculated for all the samples.

The "desorption samples" were weighed to establish their *in vivo* mass and were then placed in their respective environments. The masses were recorded daily for ten days until an equilibrium mass was reached. This was the hydrated mass of the samples. These were subsequently dried over phosphorus pentoxide for ten days and, again, the hydrated regains were calculated.

The mean values determined at each relative humidity for both sorption and desorption data were then plotted against the respective relative humidities to produce a sorption and desorption isotherm.

4.3.4 Hysteresis

Relative hysteresis was calculated on a dry matter basis as (desorption - sorption) x 100 and then divided by sorption (Jeffries 1960a).

4.4 Results

4.4.1 Hydrated Moisture Content of Donkey Hoof Horn - Full Hoof Wall Depth Samples

The results for hydrated moisture contents were compared over the seven days of saturation of samples to ascertain how quickly samples attained full hydration. There were no significant differences between the results for any of the seven days ($p>0.05$, Kruskal-Wallis). Table 4.2 shows the hydrated moisture content and hydrated regain for full HWD samples of donkey hoof horn.
donkey hoof horn. Both data sets showed a normal distribution ($p>0.05$).

Table 4.2 - Donkey Hoof Horn Full Hoof Wall Depth Samples - Hydrated Moisture Contents (n=5)

	Hydrated Moisture Content (%)	Hydrated Regain (%)
	38	62
	38	61
	39	65
	39	65
	39	65
Mean	39	64
SD	1	2
CV (%)	2	3
Normality?	Y	Y

4.4.2 Hydrated Moisture Contents of Horse Hoof Horn - Full Hoof Wall Depth Samples

Table 4.3 shows the hydrated moisture content and hydrated regain for full HWD samples of horse hoof horn. Both data sets showed a normal distribution ($p>0.05$). The full data sets are shown in Appendix 7.

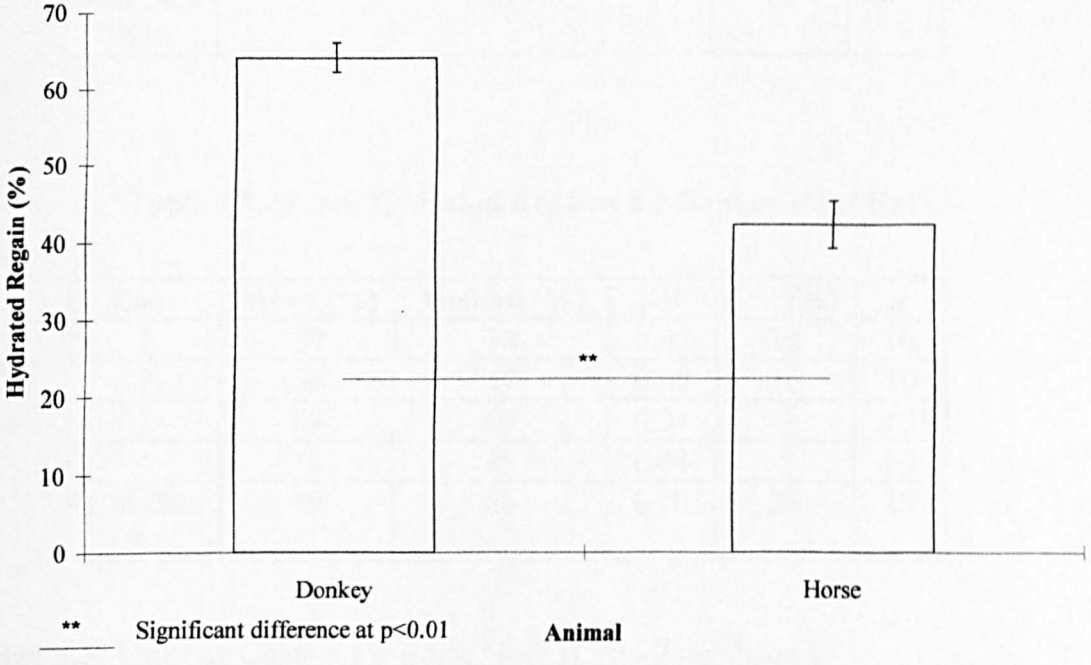
Table 4.3 - Horse Hoof Horn for Full Hoof Wall Depth Samples - Hydrated Moisture Contents (n=16)

	Hydrated Moisture Content (%)	Hydrated Regain (%)
Mean	30	42
SD	1	3
CV (%)	5	7
Normality? (p>0.05)	Y	Y

4.4.3 Comparison of Hydrated Moisture Contents Between Donkey and Horse Hoof Horn

There was a significant difference between the hydrated regain for donkey and horse hoof horn ($p<0.01$, Student's *t* test) (Figure 4.1).

Figure 4.1 - Comparison of Hydrated Regain Between Donkey and Horse Hoof Horn



4.4.4 Hydrated Moisture Contents for Donkey Hoof Horn - Zonal Samples

The zonal hydrated moisture content and hydrated regain are shown in Table 4.4 and Table 4.5 respectively. The full data sets are shown in Appendix 8. The zonal data for both hydrated moisture content and hydrated regain showed a normal distribution. There was a significant difference for hydrated moisture content and hydrated regain between all zones ($p<0.01$, One way ANOVA). The Tukey test indicated there were significant differences between a combination of all zones, except for between zones 3 and 4.

Table 4.4 - Zonal Hydrated Moisture Content Results for Donkey Hoof Horn

Zone	Mean (%)	Median (%)	SD	CV (%)	*n*
1	27	28	2	9	10
2	36	36	4	10	10
3	41	41	1	3	10
4	43	43	1	3	10
Full Data Set	37	40	7	18	40

Table 4.5 - Zonal Hydrated Regains for Donkey Hoof Horn

Zone	Mean (%)	Median (%)	SD	CV (%)	*n*
1	37	38	0.05	12	10
2	56	47	0.09	16	10
3	69	69	0.04	5	10
4	75	75	0.04	5	10
Full Data Set	59	66	0.16	26	40

4.4.5 Hydrated Moisture Content for Horse Hoof Horn - Zonal Samples

Table 4.6 shows the zonal hydrated regain for horse hoof horn. The full data set is shown in Appendix 9. The hydrated regain for all zones showed a *non*-normal distribution ($p<0.05$). There was a significant difference for hydrated regain between

all zones ($p<0.01$, Kruskal-Wallis test). A Mann-Whitney U test showed there was no significant difference between hydrated regain for zones 1 and 2 ($p>0.05$) but there were significant differences between the combinations of all other zones ($p<0.01$).

Table 4.6 - Zonal Hydrated Regain for Horse Hoof Horn

Zone	Mean (%)	Median (%)	SD	CV (%)	*n*
1	40	37	9	27	16
2	40	37	6	23	16
3	46	45	9	15	16
4	62	61	11	20	16
Full Data Set	47	43	13	17	64

4.4.6 Comparison Between Zonal Samples of Donkey and Horse Hoof Horn

There were significant differences between all zones for median hydrated regain for donkey and horse hoof horn ($p<0.01$, Mann-Whitney U tests).

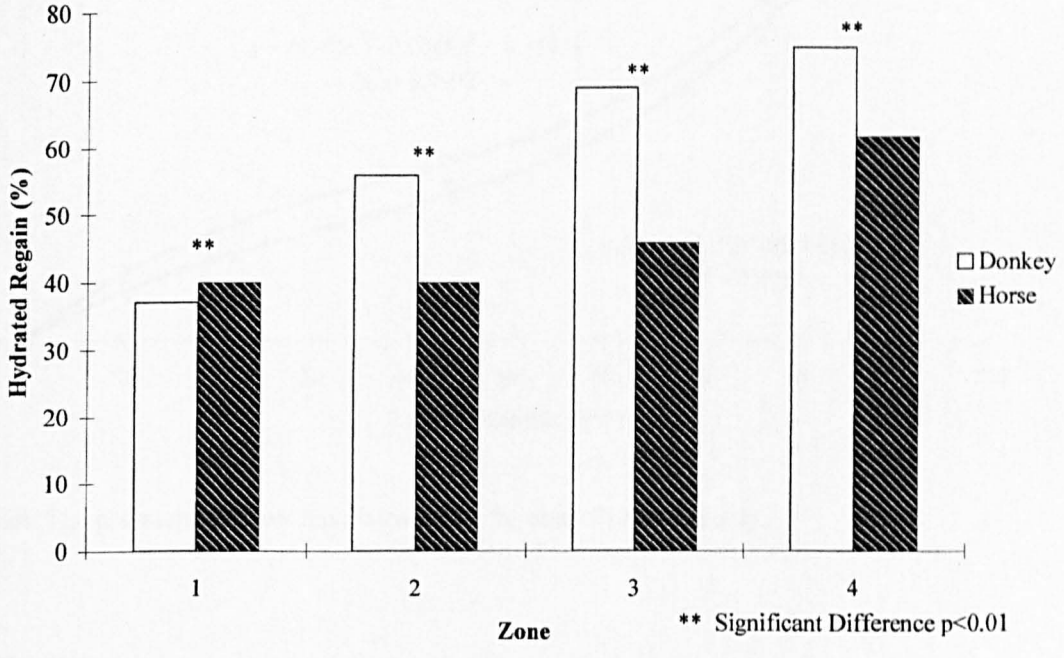
The results for percentage saturation at which the hoof wall functions for both donkey and horse hoof horn are shown in Table 4.7 for both full HWD and zonal samples. There was no significant difference between the percentage saturation of the full HWD ($p>0.05$, Student's t test) or for each zone for both donkey and horse hoof horn ($p>0.05$, Kruskal-Wallis).

Table 4.7 - Median Percentage Saturation at Which the Hoof Wall Functions for Both Donkey and Horse Hoof Horn

	Donkey (%)	Horse (%)
Full Hoof Wall Depth	91	96
Zone 1	95	92
Zone 2	94	94
Zone 3	95	95
Zone 4	93	90

There was no significant difference between any zones for donkey hoof horn ($p>0.05$, one way ANOVA). There were no significant differences between zone 1 and zone 4 and between zone 2 and zone 3 for horse hoof horn ($p>0.05$, Mann-Whitney *U* tests) but there were significant differences between the combinations of all other zones ($p<0.05$, Mann-Whitney *U* tests).

Figure 4.2 - Comparison Between Median Hydrated Regain for Zonal Samples of Both Donkey and Horse Hoof Horn

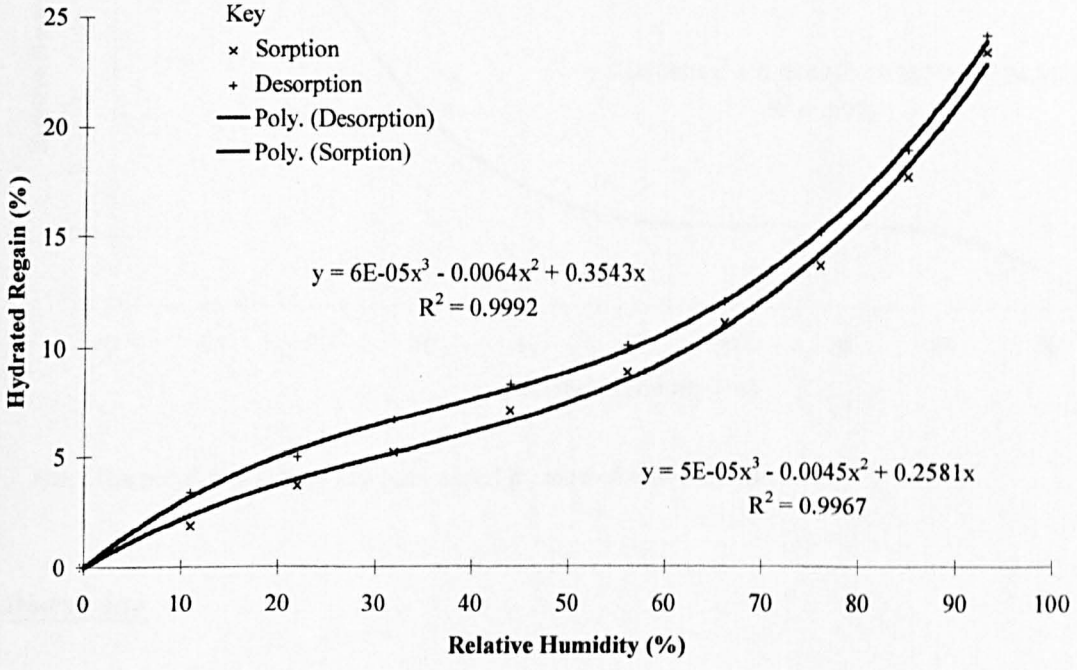


4.4.7 Sorption and Desorption Isotherms

The sorption and desorption isotherms for donkey hoof horn equilibrated using saturated salt solutions for both the sorption and desorption isotherms are shown in Figure 4.3. An increase in relative humidity produced an increase in hydrated regain. There was no significant difference between the hydrated regain of samples prior to being placed in the individual environments ($p>0.05$, Kruskal-Wallis).

At high humidities the samples achieved equilibrium mass after six days within their respective environments. However, samples at lower humidities achieved equilibrium after a few days. Room temperature was approximately 23°C.

Figure 4.3 - Sorption Desorption Isotherms for Donkey Hoof Horn

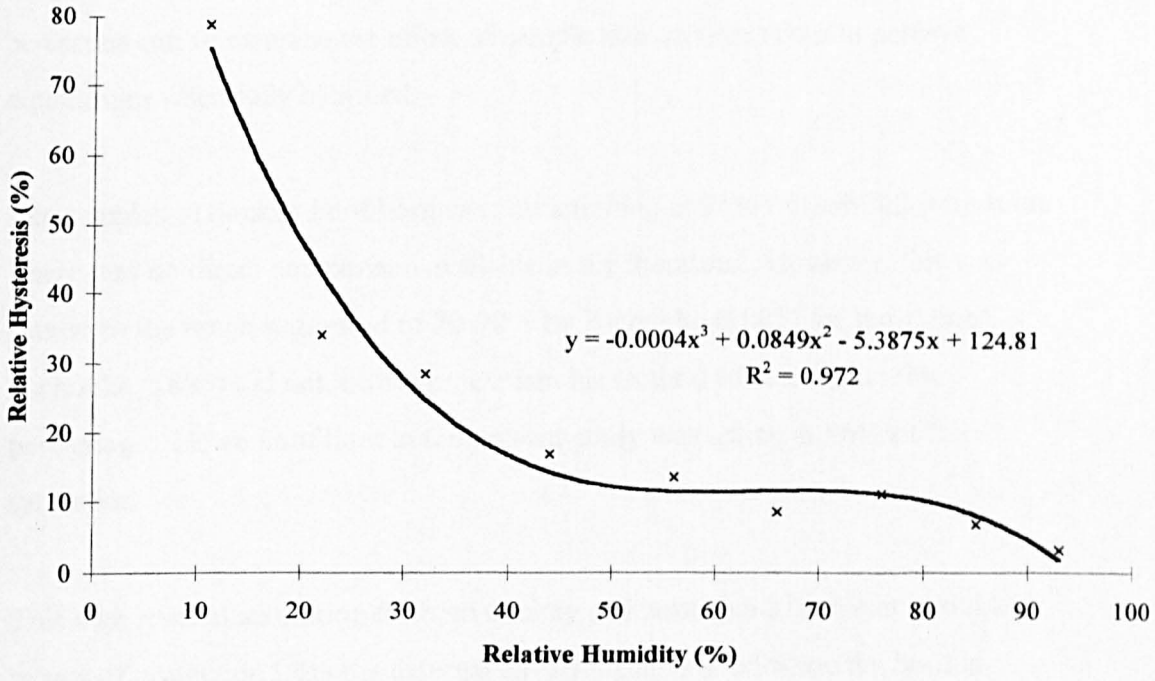


NB: The polynomial curves have been added for ease of reference only.

4.4.8 Hysteresis

Mann-Whitney *U* tests revealed there were significant differences between sorption and desorption samples for all environments ($p<0.05$) except for those at 93% RH ($p>0.05$) resulting in the relative hysteresis decreasing as the relative humidity increased (Figure 4.4). Figure 4.4 indicates a 79% relative hysteresis for 11% RH and a 3% relative hysteresis for 93% RH.

Figure 4.4 - Relative Hysteresis as a Function of Relative Humidity for Donkey Hoof Horn



NB: The polynomial curve has been added for ease of reference only.

4.5 Discussion

4.5.1 Hydrated Moisture Content for Full Hoof Wall Depth

Although there were slight increases in sample mass of donkey hoof clippings over the initial seven day period of hydration, there was no significant difference between hydrated moisture contents from day 1 to day 7. Equilibrium mass was achieved after one day. However, samples were hydrated for seven days as a safeguard to ensure full sample hydration as, for human *Stratum corneum*, Scheuplein and Morgan (1967) and Anderson *et al* (1973) found that several days were necessary to allow for equilibration of the sample. Naumann (1984) hydrated horse hoof samples for twelve days and water absorption beyond this time was not noted. Reilly (1999) hydrated samples of horse hoof for seven days until equilibration but these were much larger samples. Sample size may therefore be important and should be taken into account when hydrating large samples as water has to penetrate to a greater

depth than in small samples. It is therefore recommended that samples are weighed daily until they achieve an equilibrium mass. Further investigation should therefore be carried out to examine the effect of sample size on time taken to achieve equilibrium when fully hydrated.

The samples of donkey hoof horn were functioning at 91% of their full saturation. There was no direct comparison available in the literature. However, this was similar to the range suggested of 70-90% by Zschokke (1885) for horse hoof. Zschokke (1885) did not, however, explain his method of calculating this percentage. Horse hoof horn in the present study was acting at 96% of full saturation.

This high level of saturation for both donkey and horse hoof horn may provide a means of protection from the external environment as even when the hoof is submerged in water, it only has the ability to absorb a minimal amount of water. This would therefore prevent catastrophic failure from an oversaturation of the hoof.

As samples of hoof horn are highly saturated, a very efficient barrier to moisture loss must therefore exist for a hoof to have such capabilities. Or indeed, a very efficient replenishment system must exist if hoof, *in vivo*, continually loses moisture to the external environment.

Naumann (1984) found a slightly higher fully hydrated moisture content of 37% for horse hoof than the 30% found for donkey hoof horn in this study. However, the method of calculation of hydrated moisture content was not indicated and Naumann (1984) may well have reported hydrated regain and not hydrated moisture content.

Bertram and Gosline (1987) found a hydrated regain of 40.2% for fully hydrated horse hoof, which is much lower than that found in this study for donkey hoof horn of 66% for full HWD samples. The median result calculated from the data for hydrated regain found by Reilly (1999) for pony hoof horn was 35%. It is very unlikely that the method of calculating dry weight used by Reilly (1999) of

equilibrating samples at room temperature would account for this great difference as only a 7% difference was found between drying samples at room temperature and drying them over phosphorus pentoxide in this present study (Chapter 2). His results were slightly lower than the 42% found for horse hoof horn in the present study. Apart from differing sample sizes and different methods of hoof horn dehydration between both studies, there is no clear explanation for these differences.

The comparison of the results for donkey hoof horn with those from horse hoof horn from other authors and, indeed, from this study, indicates that there is indeed a difference between absolute values of hydrated regain for donkey and horse hoof horn. The reason for this difference is unknown and would need to be investigated though it may be as a result of differences in tubule morphology or protein content of the hoof wall.

4.5.2 Hydrated Moisture Content for Zones

Although the actual values for hydrated moisture content vary between most zones for donkey hoof horn, nearly all zones act at similar levels of saturation which may be an optimum level for this material.

For donkey and horse hoof it has been shown that each zone has similar capabilities for the uptake of moisture. This may act as a self regulating mechanism as a different uptake of water across the zones may drastically change the mechanical properties of each zone and thereby alter the function of the hoof.

There was no significant difference between the hydrated regain for zones 3 and 4 for donkey hoof horn which may have been expected as there was also no significant difference between actual moisture contents for these two regions. However, there was a trend to an increasing hydrated moisture gradient in a dorso-palmar direction across the full hoof wall depth.

A comparison of the results between all zones for hydrated regain for donkey and horse hoof horn indicated there were significant differences between zones.

Although zone 1 results appeared to be similar to each other, the results from the remaining zones were considerably different. It was also interesting to note that the outer two zones of horse hoof samples had the same hydrated regain which was different to the gradual dorso-palmar increase in hydrated regain for donkey hoof horn (Figure 4.2). Reasons for these differences are unclear and need to be investigated. This result does, however, endorse the differences that have already been shown between hydrated regain for full HWD between donkey and horse hoof horn.

In contrast to there being no significant difference for hydrated regain for zones 3 and 4 for donkey hoof horn, there were significant differences for all zones for horse hoof horn except between zones 1 and 2. This mirrored the results shown for moisture content in Chapter 3. The reason for this lack of a difference is unclear but may depend on, for example, tubule density, tubule morphology or protein content of the hoof.

A comparison of Kasapi and Gosline's (1997) results for fully hydrated samples with those of hydrated moisture content for donkey hoof horn from this study indicated that horse hoof takes up more water than donkey hoof in zones 1 and 4 but the middle zone for horse hoof is similar to zone 3 in this work. Kasapi and Gosline (1997) examined three zones, an outer, middle and inner zone which had full hydration levels of 35%, 41% and 48% respectively. However, they did use different drying techniques and dried samples at 100°C for five days and did not indicate their method of calculating hydrated moisture content. This different drying technique would not account for the differences in hydrated moisture content as a higher drying temperature would decrease the dry mass and, in turn, result in an increased hydrated moisture content. However, the mean of their three sample sites of 41% is only slightly higher than that found in this study for full HWD samples for donkey hoof horn. However, their samples were only taken from one animal.

The hydrated moisture content results for horse hoof horn in the present study were considerably lower than those from Kasapi and Gosline (1997) and Kasapi (1997).

Again, as outlined above, differences in methodology or calculations may account for these differences.

Kasapi and Gosline (1997) and Kasapi (1997) suggested their zonal differences in hydrated moisture contents may be partly due to tubule morphometry as the medullary cavities of middle and outer wall tubules occupy 1.5% and 4.5% of the hoof wall area respectively, resulting in less *Stratum medium* being available in the outer wall to absorb water and hence resulting in a lower water content. Reilly (1999) found similar sizes for medullae to these other authors but these have not yet been calculated for donkey hoof horn. The effect of the size of medullae on moisture content of hoof horn should be investigated further.

Kasapi and Gosline (1997) and Kasapi (1997) also believed that differences in protein type and content may also contribute to the differences in moisture content but these comments were not substantiated from their experimental results.

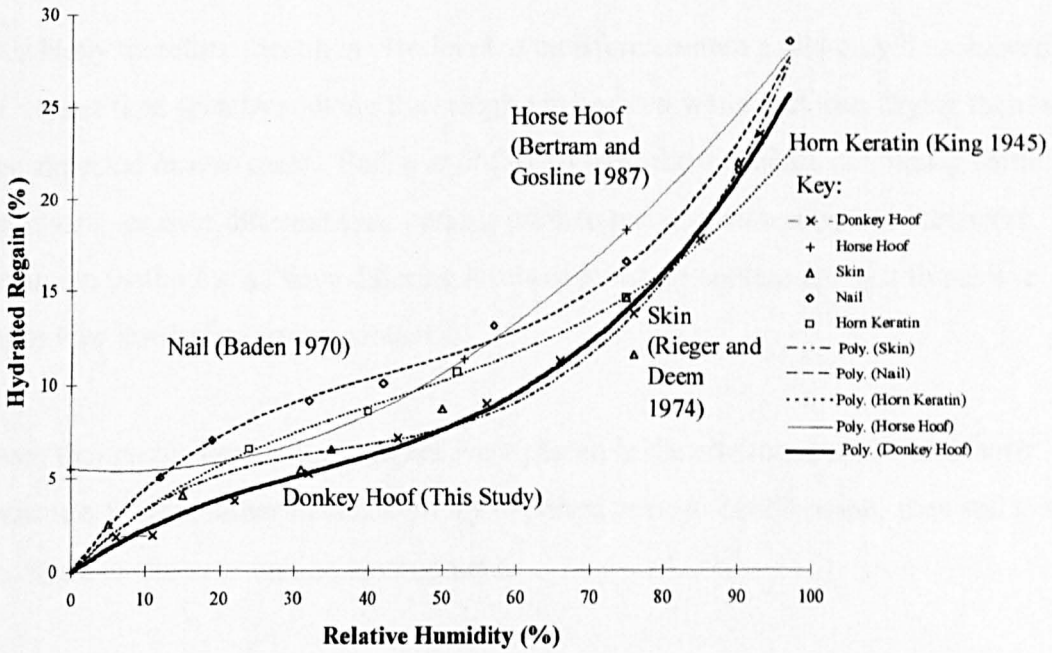
As shown in Chapter 2, and has been reiterated in this section, a lack of standardised protocols throughout the literature provides difficulties in comparison of results with previous work. The utilisation of hydrated moisture content and hydrated regain provides a means of manipulating the moisture content of hoof horn when there have been storage problems or where the fresh mass is unknown.

4.5.3 Relative Humidity Environments

The results from samples that were equilibrated over saturated salt solutions showed a typical sigmoidal shape sorption isotherm and were similar to that shown for other keratinised tissues such as horse hoof, horn keratin, skin and nail (Bertram and Gosline 1987; King 1945; Rieger and Deem 1974; Baden 1970 respectively) (Figure 4.5). The desorption isotherm was also sigmoidal and was similar to that shown for horn keratin (King 1945) and for wool (Speakman 1930). The desorption isotherms were not shown for skin, nail and horse hoof.

It was initially believed that the results from the sorption and desorption isotherms would provide an indication of the level of relative humidity required to rehydrate samples to their *in vivo* level. Samples at low humidities appeared to equilibrate after three days whereas those at high humidities equilibrated after six days. King (1945), Baden (1970), Rieger and Deem (1974) and Bertram and Gosline (1987) did not indicate whether or not their samples reached an *in vivo* moisture content. However, in the present study it was still not possible to reproduce a hydrated regain of 42% which was equivalent to an *in vivo* moisture content of 33% necessary to rehydrate samples.

Figure 4.5 - Comparison of Donkey Hoof Horn Sorption Isotherm with Other Keratinised Tissues



NB: The polynomial curves were added for ease of reference only.

The reason for this may have been that it was not possible to record very slight differences in mass over time and a true equilibrium for samples stored in high humidity environments may not be expected for an extremely long period of time. This may have happened as the rate at which absorption of water occurs is

determined by the concentration which, in the vapour phase, is very small. The absorption of water into the sample in the vapour phase would therefore be very small.

D'arcy and Watt (1970) believed that full saturation is extremely difficult to obtain via humidity environments, although for wool, the time required to attain equilibrium has been shown to be ten days (Bull 1944). For human *Stratum corneum*, Anderson *et al* (1973) found that samples stored in environments above 80% RH took a much longer time to equilibrate. Longer periods of equilibration time were not employed in this present study because of sample deterioration at high relative humidities. The hoof samples used in the present study were thicker than both wool or *Stratum corneum* and may therefore require a longer equilibration time owing to the greater distance for penetration of the water molecules into the sample.

It is likely therefore that an *in vivo* level of moisture content could only be achieved in a short time span by soaking the samples in distilled water and then drying them to the expected *in vivo* mass. Baillie *et al* (2000) used the technique of soaking cattle hoof samples over different time periods prior to mechanical testing but they were using the method to achieve differing levels of moisture content and not to achieve an *in vivo* level of moisture content.

Even though the desorption samples were placed in the environments at an *in vivo* moisture content rather than being fully hydrated prior to equilibration, they still lost moisture to the surrounding environment.

It was not known initially whether the moisture content of the samples taken from the MDC section would have similar moisture contents. However, there were no significant differences between the moisture content of samples from the MDC region showing that there was no medio-lateral gradient in hoof moisture content for this particular area.

The main polar groups in a protein such as keratin are the free amino, carboxyl, and hydroxyl groups of the amino acid side chains. Between 0% and 20% relative humidity the equilibrium water uptake increased rapidly, corresponding to binding of water to highly active sites and indicating the amount of strongly bound water molecules (Lévêque 1994). For wool, at low water contents, water is mainly tightly bound to the carboxyl and amino groups (Leeder and Watt 1965; Watt and Leeder 1968). As water content increases, these tight binding sites become occupied and water then binds to weaker binding sites and to water which is already bound (Watt and Leeder 1964; Fennema 1972; Nissan 1976). In wool, this showed a change from a monolayer of water molecules to multilayer coverage (D'arcy and Watt 1970) together with an increased interaction with other molecules (Mark *et al* 1987). Water uptake was approximately proportional to the increase in relative humidity up to 80%. Once all the binding sites are occupied, water is absorbed as bulk water. Similar observations may be expected with hoof horn based on its similar keratin structure.

The comparison of results between sorption and desorption at different levels of relative humidity indicated that there was in fact hysteresis as the values for desorption were significantly greater than those for sorption except at 93% RH. This meant that a 7% moisture content for the sorption isotherm was equivalent to a 50% RH, whereas for the desorption isotherm it was equivalent to 39% RH, a difference of 11%, *i.e.* a change of 11% in relative humidity is required for hoof to pass from the sorption to the desorption condition, or vice versa. There appears to be a dispute over this value for wool. D'arcy and Watt (1981) found it to be as low as 3% whereas Von Bergen (1963) found it to be much higher at 18%. The relative hysteresis for the full sorption and desorption isotherms does not appear to have been calculated for other keratinous materials. However, for celluloses, the relative hysteresis decreased with increasing relative humidity (Meredith 1957; Jeffries 1960a,1960b) which was similar to that found for this present study. Hysteresis in natural materials is likely to have a particular function. In nature, hysteresis may be considered as a built-in protective mechanism against extremes, such as loss of water

due to a dry atmosphere (Kapsalis 1981). This may therefore be extremely useful for donkeys living in hot climates.

As the difference between sorption and desorption values is high at various levels of relative humidity for donkey hoof horn, it is important to know whether the direction is one of sorption or desorption when using relative humidity environments to equilibrate samples as an 11% difference in moisture content may result in a very different mechanical testing result as moisture is known to affect the mechanical properties of hoof horn. There is no mention in the previous studies on horse hoof as to whether equilibration was actually achieved or whether this was achieved by sorption or desorption.

Bertram and Gosline (1987) believed that an environment of 75% RH provided the equivalent of an *in vivo* moisture content for horse hoof. However, from these present results it can be seen that there were significant moisture losses for donkey hoof horn at 76% RH. Their subsequent mechanical tests indicated that fully saturated hoof horn was more liable to crack propagation than that at 75% RH. This may well have been the case but, according to the results found in this study, the *in vivo* moisture content would have been considerably higher and therefore there may not be a great difference between crack propagation for *in vivo* and fully hydrated samples.

The results reported in this thesis indicate that the practice of placing samples in 97% RH to avoid bulk water accumulating in the medullary cavities of the tubules which was carried out by Kasapi and Gosline (1997) and Kasapi (1997) would result in a considerable loss in moisture content if samples of horse hoof behave similarly to donkey hoof. Considerable moisture loss may also have been seen by Wagner *et al* (2001) who maintained capsules overnight in a 41% RH environment.

The hydrated regains from this present study following equilibration of samples at specific relative humidities are lower than those indicated for horse hoof. These differences may be partly due to the methods used for drying samples to ascertain

moisture contents or to the species difference. The drying techniques must therefore be standardised.

4.5.4 Future Work

Similar to moisture content, a "water map" of the hydrated moisture contents around the hoof capsule of both donkey and horse hoof horn would allow investigation into how the hoof copes with considerable wetting.

Possible reasons for the existence of a similar hydrated regain between zone 1 and 2 for horse hoof should be investigated as this did not exist for donkey hoof horn.

The effect of size of tubule medullae on the moisture content of donkey hoof horn should be examined as alterations in the sizes of tubules and their components may be a way that moisture content of the hoof can be manipulated.

The influence of sample size on the time taken for samples of hoof horn to equilibrate should be examined.

Hydration experiments should be carried out on horse hoof horn to see whether there are, indeed, species differences between the sorption and desorption of water into or out of hoof horn.

4.6 Conclusions

- Samples can be saturated for one day to ascertain fully hydrated mass and then dried over phosphorus pentoxide to ascertain dry mass in order to calculate hydrated moisture content. It is suggested, however, that samples are hydrated until an equilibrium mass is achieved.

- Hydrated moisture content is useful for providing absolute values for the amount of water present in a saturated sample of hoof.

- Hydrated moisture content and hydrated regain avoid the introduction of *in vivo* fresh weights into calculations. This provides less variability in results.

- Donkey hoof horn functions at 91% of its full saturation level. Each zone also operates close to its saturation point.

- Horse hoof horn functions at 96% of its full saturation level. Each zone also operates close to its saturation point.

- There was no medio-lateral moisture content gradient for the samples that were tested.

- The hydrated regain for donkey hoof horn was shown to be significantly higher than that found for horse hoof horn, indicating a species difference.

- There were significant differences between hydrated regain for zones for donkey and horse hoof horn, again indicating a species difference.

- The method of fully hydrating samples of hoof horn will be used for samples that are tested mechanically in Chapter 6.

- It was not possible to reproduce an *in vivo* moisture content following sample equilibration at high humidities during the time allowed for equilibration. Longer timescales involved deterioration of samples.

- The use of relative humidity environments to equilibrate samples is still a useful method of rehydrating samples and normalising moisture content. However, any reporting of mechanical testing results following equilibration of hoof horn samples at different relative humidities should also include the moisture contents of the samples.

- The sorption and desorption isotherms provided a useful method of indicating the relationship between the moisture content of hoof and relative humidity environments;

- The hysteresis shown in the isotherm results indicated that it is necessary to know whether the moisture content of samples following equilibration in a particular relative humidity environment has been achieved either by sorption or desorption as this will alter the value for moisture content.

- The use of sorption and desorption isotherms does not provide a means of ascertaining the moisture content of horse hoof horn samples equilibrated at various relative humidities by other authors as the results for donkey hoof horn were lower.

- The sorption isotherm for donkey hoof horn showed similar results to other keratinised tissues such as wool, horn, skin and nail.

- The desorption isotherm for donkey hoof horn showed similar results to other keratinised tissues such as wool and horn.

- Relative humidity environments will be used in Chapter 6 and Chapter 7 to hydrate samples of donkey hoof horn in preparation for mechanical testing.

5. TUBULE DENSITY

5.1 Introduction

The review of literature in Chapter 1 has shown that there is very little information about the structure of donkey hoof horn. One method of providing quantitative information is to assess the tubule density of hoof horn. This has not been reported for donkey hoof horn until this present study.

As has been outlined in the review of literature, tubule density is believed to influence hoof function and the mechanical properties of hoof horn, hoof quality, moisture content and can be used for comparative purposes between species.

Tubule density may also be used in the future to ascertain hoof quality. As the "normal" tubule density for the *Stratum medium* of donkey hoof horn was established in this thesis, this will allow a comparison of hoof horn from animals kept under different management regimes or those with foot problems.

Many of the comments made in the literature on the importance of, and effect of, tubules are, however, still unsubstantiated (Balch *et al* 1997; Schummer *et al* 1981; Bertram and Gosline 1986) and many comments continue to be "cast in stone". An example of this is from Balch *et al* (1997) who stated that the orderly proximodistal arrangment of tubules and intertubular horn provides strength to the hoof wall.

Reilly *et al* (1998b) suggested that the results of their study together with the results of Reilly *et al* (1996) pointed to a four zone pattern of tubule density at the MDC of the *Stratum medium* of pony and horse hoof that may represent an equine pattern. If so, this would also exist for donkey hoof horn.

The use of clippings for studies on hoof horn has been discussed in Chapter 1. In this study, comparisons were made, as far as was reasonably possible, between the use of

clippings and morbid hoof horn to ascertain tubule density. The actual measurement of HWD has not been reported for donkey hoof horn. This was also investigated as there may be a relationship between HWD and tubule density as the thickness of the HWD may be a supportive mechanism by which the animal may cope with its own bodyweight. Bodyweight and age are two of the many factors which are also thought to influence tubule density. Although these two factors were not incorporated specifically into the experimental design, they were thought to be interesting and were therefore also examined in this chapter.

The influence of the presence of tubules on moisture content is in dispute (Chapter 2). As moisture content has been shown to influence the mechanical properties of hoof horn then if tubule density does, indeed, influence the moisture content of hoof horn, differences in tubule density across the HWD may reflect differences in moisture content across this area and may result in different mechanical properties across the HWD.

5.2 Aims

The aims of this chapter were:

- to quantify the tubule density at the midline dead centre for the *Stratum medium* of donkey hoof horn;

- to use the results to test for the presence or otherwise of a four-zoned pattern that had previously been identified for pony and horse hoof;

- to assess the relationship between hoof wall depth and tubule density;

- to compare the use of clippings with the use of morbid samples to establish tubule density;

- to use the results to study the inter-relationships between tubule density, mechanical properties and moisture content of donkey hoof horn (Chapter 8).

5.3 Materials and Methods

The samples of clippings used in the investigations described below were obtained from the animals identified in Chapter 2. Preliminary investigations to ascertain tubule density were carried out on donkey hoof clippings. Further work was carried out using samples from morbid donkey hoof capsules.

5.3.1 Clippings

The samples used in this part of the study were the same samples (n=16) taken from clippings that were used for mechanical testing (Chapter 6) (Appendix 1). Samples of 30 mm in length were taken from the MDC and the white lines were removed using a scalpel. The samples were milled perpendicular to the direction of the tubules using a Minicraft sander[5]. During sanding the sample was attached to a small piece of wood by Bostik Blu-Tack[6] to enable it to be held against the sander. The top surface of the samples was marked using an indelible pen. The resultant sections were approximately 2 mm in depth, 30 mm in length with the width dictated by the dimension of the HWD. The depth was measured using vernier calipers. The samples were then wrapped in Parafilm and stored in a refrigerator at 4°C.

Perpendicular sectioning of tubules was essential to reveal accurate tubule density data. Sections not cut in this way may lead to inaccuracies from incorrect cross sections.

Following mechanical testing, a block was removed from the centre of the 30 mm sample having a 10 mm mediolateral width, thus still corresponding to the MDC of the original clipping. Samples were mounted onto small wooden blocks using

[5] MB450, Minicraft, Roto Zip UK Ltd, Durham.
[6] Bostik Ltd, Leicester.

Araldite[7] glue which was left to set for approximately twenty minutes. Sections of approximately 12 μm thickness were removed from the top of the block using a sledge microtome. Samples were stained in Haematoxylin and Eosin, dehydrated and mounted on a slide in DPX with a coverslip.

5.3.2 Morbid Donkey Hoof Horn

Hoof horn samples were taken from the forefeet of a random sample of nine donkeys that had been humanely destroyed for reasons other than this project and had been stored at -20°C. The detail and background histories of the animals were not known. As mentioned in the literature review, the availability of hoof horn samples from morbid donkey hoof capsules is very limited. It was decided to use these samples for comparison of tubule density results between clippings and morbid samples but not to use them for moisture content or mechanical analyses as the effect of freezing on these factors, together with the full collection and storage history was not known.

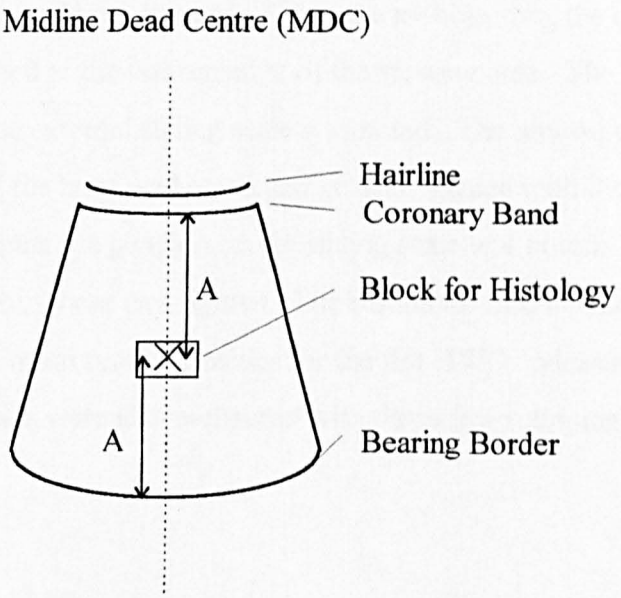
The sample site was the midline dead centre of the *Stratum medium* (after Reilly *et al* 1996). The proximo-distal position of the sample was at the mid-point between the coronary border and the bearing border, referred to as 50% HWH, and included the full HWD (Reilly *et al* 1998b) (Figure 5.1). A sample block was removed having a 10 mm mediolateral width and a HWH of 5 mm. Sections of 12 μm thickness were taken from the centre of this block using the same methodology outlined above for donkey hoof clippings.

[7] Bostik Ltd, Leicester

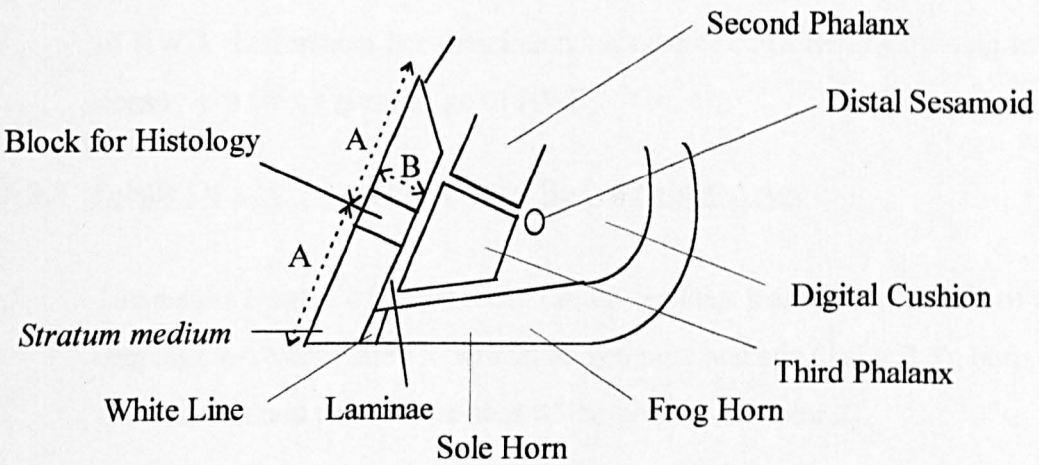
Figure 5.1: Diagrammatic Representation of the Donkey Hoof Wall Showing Position of Samples Used for Tubule Density

Key: A - Distance A is at 50% hoof wall height (HWH)
B - Hoof wall depth (HWD)

1a) Dorsal hoof Wall



1b) Sagittal Section of Hoof Wall



5.3.3 Hoof Wall Depth

Measurements of HWDs for both clippings and morbid samples were carried out on the slides. The sliding scale on the Olympus BH2[8] microscope was used to measure the entire HWD. As the hoof section was too large to be viewed entirely under a x4 objective, the usual method of measuring any sample on a microscope using an ocular and stage micrometer could not be used. Using the x4 objective, the external edge of the section was aligned at the bottom edge of the viewing area. The corresponding position on the external sliding scale was noted. The section was then moved until the inner part of the hoof wall could just be seen aligned with the top edge of the viewing area. Again the position on the sliding scale was noted. The HWD was then calculated from these two figures. The results for measurements of the HWD were compared to mean tubule densities for the full HWD. Measurements of HWDs from morbid samples were also compared with those from clippings.

5.3.4 Tubule Density

Individual slides were then placed in the negative carrier of a photographic enlarger[9] and the image was projected onto the baseboard of the enlarger. A grid of known size was then overlaid on the image and a tubule density count was carried out using the method of Reilly *et al* (1996). Thus tubule density was determined as a function of HWD. Differences between individuals were normalised by quoting tubule density at a stated percentage of HWD.

5.3.5 Tubule Density, Hoof Wall Depth, Bodyweight and Age

The results from HWDs and mean tubule densities from the full HWD of the clippings were compared to animal bodyweight and age (Table 2.3), both of which were ascertained prior to the start of the project (Chapter 2).

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[8] Olympus Optical Co (UK Ltd), London.
[9] Devere 203, Beckenham, Kent.

5.4 Results

5.4.1 Clippings

5.4.1.1 Hoof Wall Depth

The results for the comparison of HWD for donkey hoof clippings with morbid samples from 50% HWH are shown in Table 5.1. Both data sets showed a normal distribution ($p>0.05$). A one way ANOVA indicated there was no significant difference between the HWDs. However, there was a difference of 0.87 mm *i.e.* approximately 10% between the clipping samples and those taken from 50% HWH.

Table 5.1 - Hoof Wall Depth Values for Donkey Hoof Clippings and Samples taken at 50% Hoof Wall Height

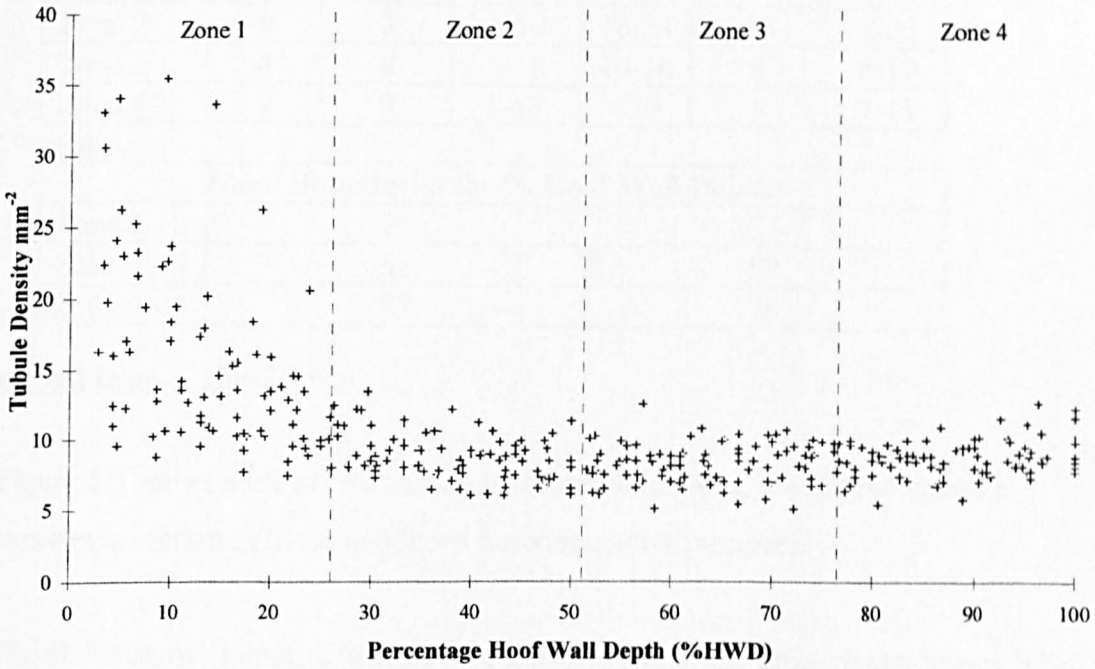
	Clippings	Unknown Animals - 50% HWH
n	16	9
Mean (mm)	7.46	8.33
Standard Deviation	1.30	1.21
Coefficient of Variation (%)	17	15

5.4.1.2 Tubule Density - Clippings

The normal probability plot showed that the data were *non*-normally distributed ($p<0.01$). However, it was not possible to transform the data to establish a normal distribution by using the square root or logarithm of the data in order to use the method outlined by Reilly *et al* (1996) and Reilly *et al* (1998b) to ascertain the boundary lines between the four zones found in their study. Therefore it was decided to use the same boundary lines as those used for pony hoof horn by Reilly *et al* (1996) for comparative purposes only. The median tubule density for the full HWD was nine tubules mm^{-2}. A scatterplot showing the decline in tubule density across the HWD from approximately thirty five tubules mm^{-2} to approximately nine tubules mm^{-2} is shown in Figure 5.2. There was a significant difference between

zone 1 and all other zones ($p<0.01$, Mann-Whitney *U* test). There was also a significant difference between zone 3 and 4 ($p<0.05$, Mann-Whitney *U* test).

Figure 5.2 - Tubule Density by Percentage Hoof Wall Depth for Donkey Hoof Horn Clippings



The results for the zonal tubule density for clippings are shown in Table 5.2. However, data for zones 1, 3 and 4 showed a normal distribution ($p>0.05$) but data for zone 2 showed a *non*-normal distribution ($p<0.01$).

Table 5.2 - (a) Comparison of Zonal Tubule Densities (TD) and (b) Zonal Divisions of the *Stratum medium* at the Midline Dead Centre for Clippings and Morbid Hoof Samples

(a)

	Clippings (Tubules mm^{-2})			Morbid Hoof Horn (Tubules mm^{-2})		
	Mean TD	Median TD	Range of TD	Zonal TD	Median TD	Range of TD
All Zones	10	9	5-35	10	10	6-34
Zone 1	16	14	7-35	>34	19	12-34
Zone 2	9	8	6-12	16-34	10	7-20
Zone 3	8	8	5-13	10-16	8	6-13
Zone 4	9	9	5-13	<10	9	7-13

(b)

Zonal Boundaries (as % Hoof Wall Depth)		
Zone 1 - 2	26	31
Zone 2 - 3	51	52
Zone 3 - 4	77	76

5.4.2 Morbid Donkey Hoof Horn

Figure 5.3 shows a transverse section of donkey hoof horn. Figure 5.4 shows a transverse section of horse hoof horn for comparative purposes.

Figure 5.5 shows tubule density by percentage HWD of the *Stratum medium* at 50% HWH at the MDC for donkey hoof horn. The results for zonal tubule density are shown in Table 5.2.

Tubule density gradually decreased in a dorso-palmar direction from ~35 tubules mm^{-2} until ~50% HWD and then remained constant at approximately nine tubules mm^{-2}, with a trend to a slight increase in tubule density towards the *Stratum internum*.

Figure 5.6 shows the data set from Figure 5.5 plotted for individual animals.

A normal probability plot indicated that the data were *non*-normally distributed ($p<0.01$). A Box-Cox Transformation, with a λ value of -1.124, was used to transform the data to provide a normal distribution.

Figure 5.3 - Transverse Section of Donkey Hoof Horn

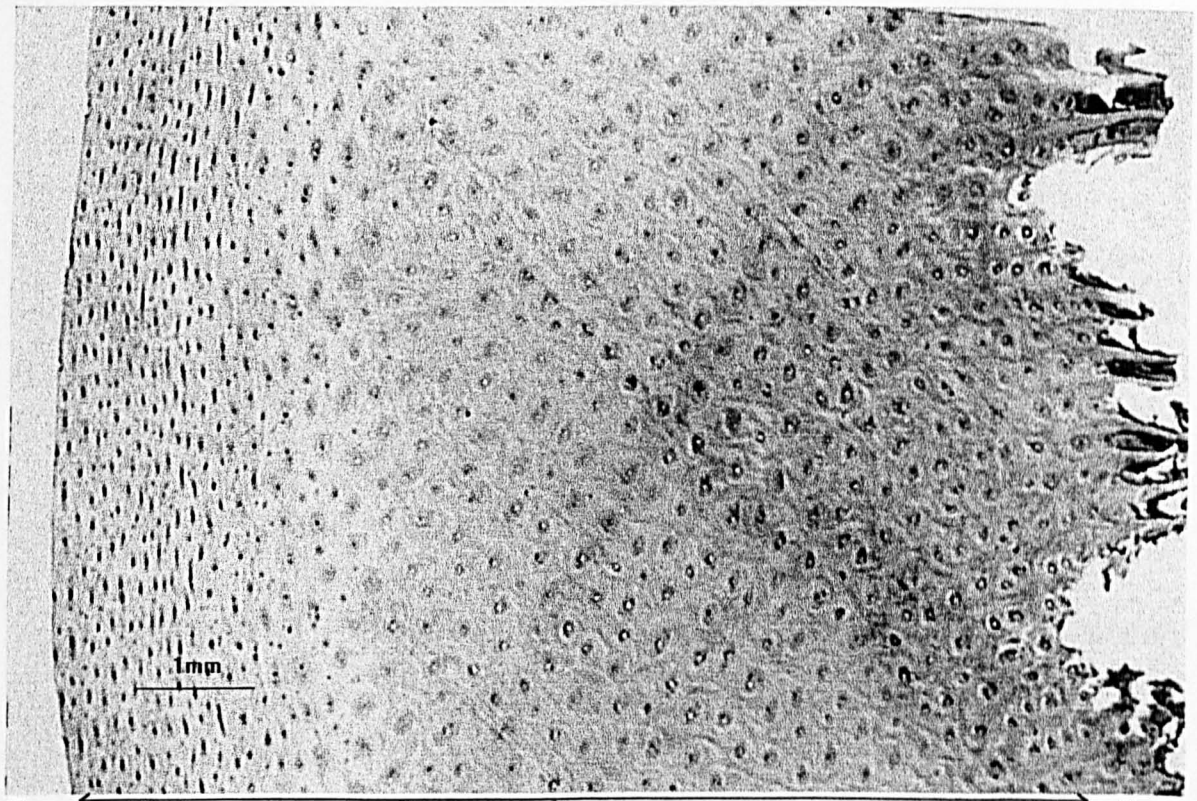
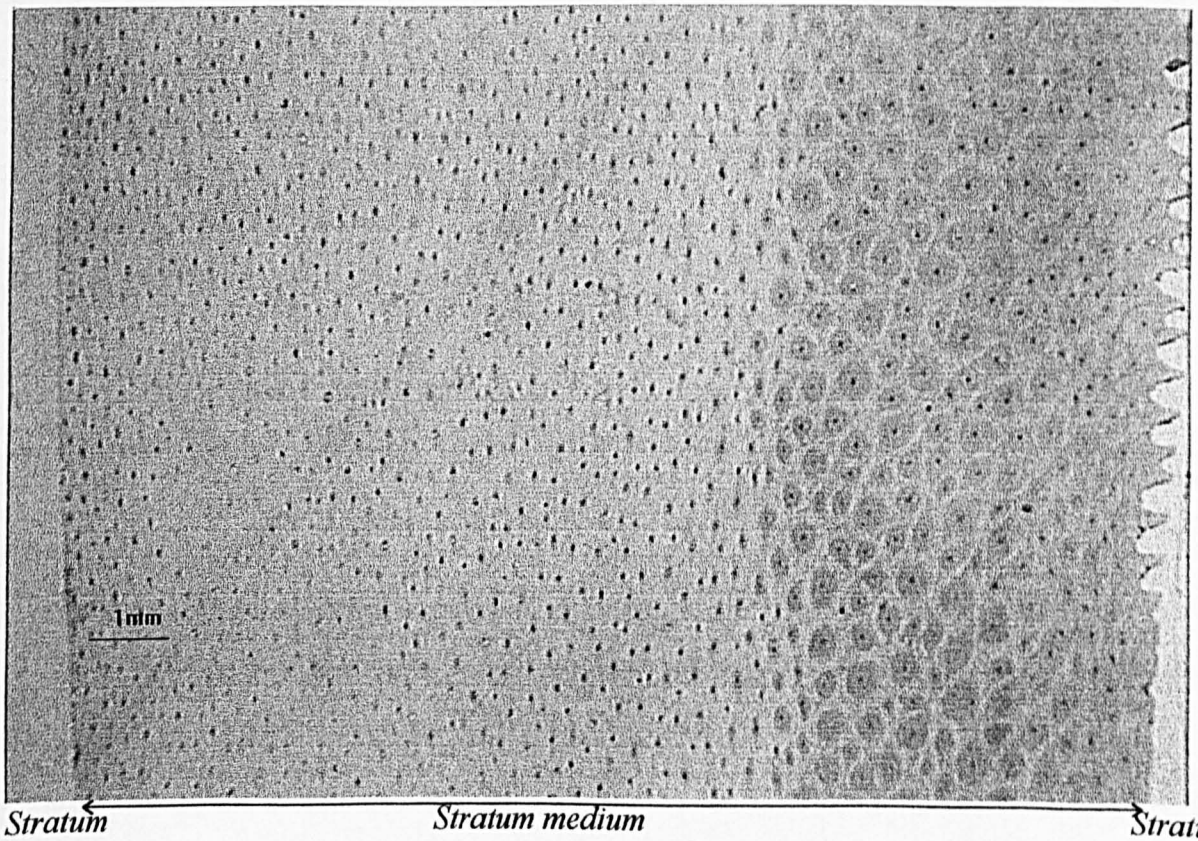


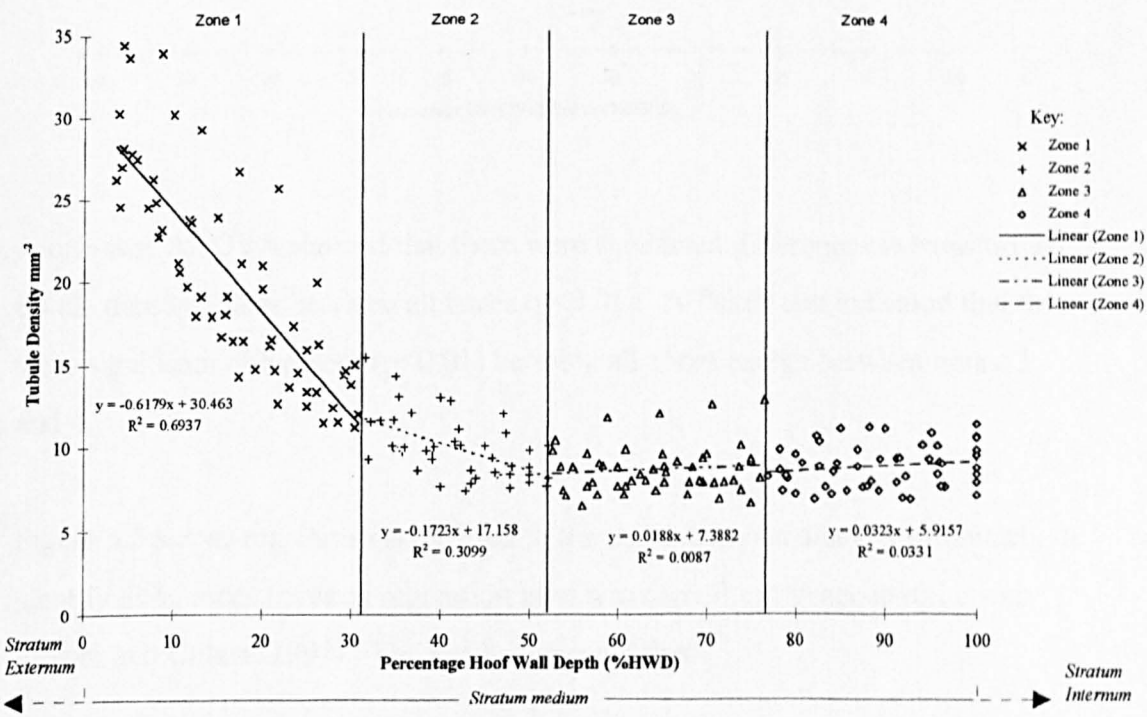
Figure 5.4 - Transverse Section of Horse Hoof Horn



Data analysis was initially carried out using the convention provided by Reilly *et al* (1996). The transformed data were divided by use of ±1 and ±2 standard deviations about the mean (0.07252 transformed tubules mm^{-2}) to provide divisions at 0.01900, 0.04576, 0.10196, 0.12872 transformed tubules mm^{-2} which correspond to tubule densities of ~34.0, ~15.6, ~7.6 and ~6.2. The mean tubule density was ~10.3 tubules mm^{-2}. These values were used to establish the %HWD at which the zonal boundaries occurred. A simple regression equation defined the relationship between %HWD and the transformed data as:-

%HWD = 811x - 6.6162 transformed tubule density

Figure 5.5 - Tubule Density by Percentage Hoof Wall Depth of the *Stratum medium* at 50% Hoof Wall Height for Donkey Hoof Horn

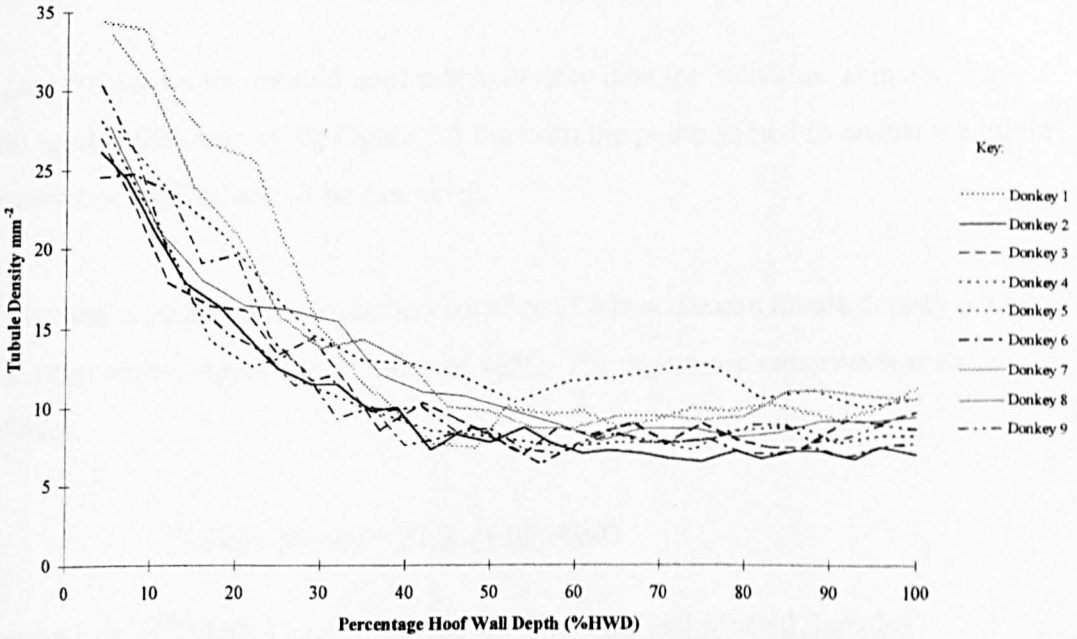


This determined the corresponding intercepts in terms of %HWD for each transformed tubule density value. Accordingly, the *Stratum medium* of the hoof wall at the MDC was then divided into four zones by the divisions at ~31%, ~52% and

~76% shown in Figure 5.5. The corresponding tubule density ranges together with median tubule density values for the zones are shown in Table 5.2.

Figure 5.6 - Morbid Hoof Tubule Density Data for Individual Donkeys

(data points are joined for ease of reference only)



A one-way ANOVA showed that there were significant differences in transformed tubule density values between all zones ($p<0.01$). A Tukey test indicated that there were significant differences ($p<0.01$) between all zones except between zones 3 and 4.

Figure 5.5 shows regression lines fitted to the zonal data. An analysis of the data to identify differences between regression lines was carried out in accordance with Fowler and Cohen (1997). The results indicated that:

- There was a significant difference between the slopes of the regression lines between zone 1 and all other zones ($p<0.01$). However, a comparison between the slopes of the regression lines for the remaining zones showed no significant difference ($p>0.05$).

- There was no significant difference between the data for zones 3 and 4, or between the slopes of the regression lines for zones 3 and 4. A comparison was therefore made between the regression lines for the combined data from zones 3 and 4 and that for zone 4 alone. The result showed there was also no significant difference between the slopes of the two regression lines.

Figure 5.6 shows the morbid hoof tubule density data for individual animals. The data used is the same as for Figure 5.5 but with the points joined to enable the tubule density from individuals to be examined.

There was no significant correlation between HWD and mean tubule density (-0.68, $p=0.066$) with a regression R^2 value of 46%. The regression equation was as follows:

$$\text{Tubule density} = 21.2 - 1.02\ \text{HWD}$$

5.4.3 Comparison of Tubule Density Results for Clippings and Morbid Samples

There was a significant difference between the overall tubule density for donkey hoof clippings and morbid samples ($p<0.01$, Mann-Whitney *U* test). Zones 1 and 2 also showed significant differences ($p<0.01$, Mann-Whitney *U* test). However, there were no significant differences between zones 3 and 4 ($p>0.05$, Mann-Whitney *U* test) (Table 5.2).

5.4.3.1 Tubule Density, Bodyweight and Age

A comparison of mean tubule density for full HWD for clippings and animal bodyweight did not show a significant correlation (-0.40, $p=0.17$). There was also no significant correlation between mean tubule density and animal age (-0.14, $p=0.64$).

There was, however, a significant correlation between bodyweight and tubule density for zone 3 (-0.651, p=0.016). A regression analysis resulted in an R^2 value of 42% (p=0.016). Although not significant, bodyweight and the tubule density from zone 2 showed a correlation of -0.551 (p=0.051).

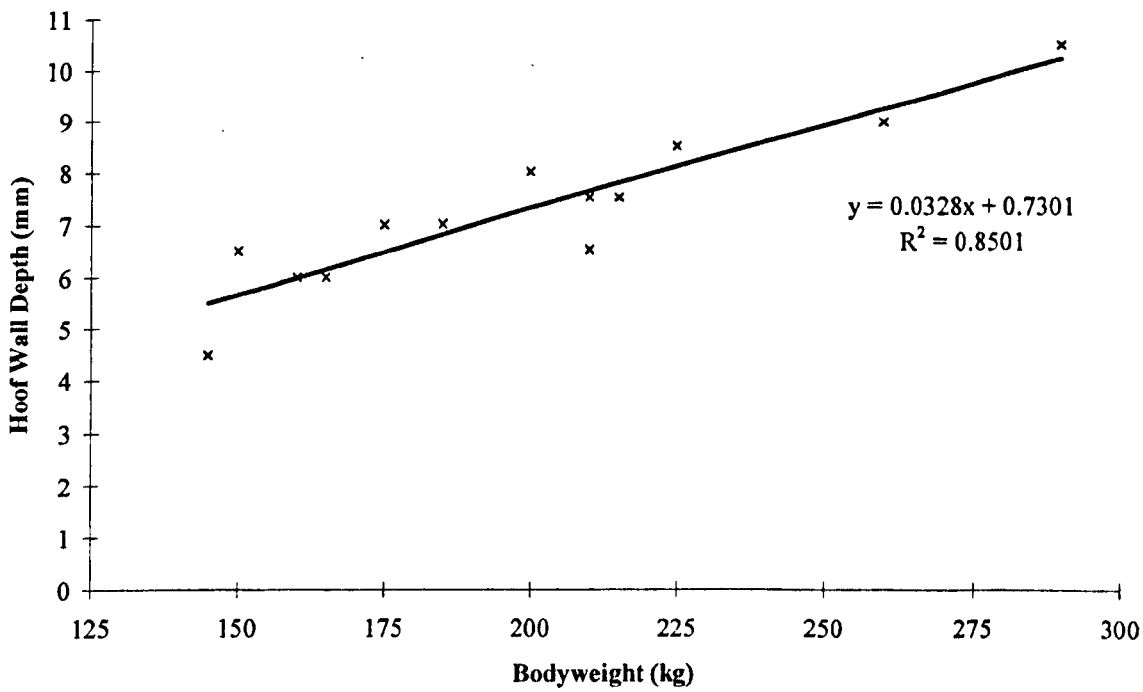
There was a very strong positive correlation between HWD and bodyweight of 0.92 (p<0.001). The relationship between bodyweight and HWD is shown in Figure 5.7. A regression analysis resulted in an R^2 value of 85% with the following equations:

Bodyweight = 11 + 25.9 HWD

HWD = 0.73 + 0.0328 bodyweight

There was no significant correlation (-0.35, p=0.24) between HWD and mean tubule density.

Figure 5.7 - Interaction of Animal Bodyweight and Hoof Wall Depth



5.5 Discussion

The same method used for tubule counting by Reilly *et al* (1996 and 1998b) and Reilly (1999) was used for comparative purposes only in this present study in order to see whether the four zoned pattern existed for donkey hoof horn. Photographs were not produced in the same way as in the above studies, but in this thesis an enlarger was used as a means of projection to avoid the costs involved in producing hard copies of images.

The morbid samples of unknown animals may, inadvertently, have been biased towards older animals. This suspected bias may have had an effect on tubule density as Geyer (1980) indicated there was an inverse relationship between tubule density and age in pigs but his study did not support this. However, using the results from clippings in the present study, there was no significant negative relationship between tubule density and age of the animals although the study was restricted to those animals of nine years of age and under.

5.5.1 General - Clipping and Morbid Samples

Tubule densities determined in this study varied across the HWD, with tubules in the outer *Stratum medium* being more numerous than those in the remainder of the hoof wall. This is consistent with measurements reported by other authors for pony (Reilly *et al* 1996; Reilly 1999) and horse hoof (Rössner 1940; Kasapi and Gosline 1997; Reilly *et al* 1998b) and may account for the higher compressive strength in the outer wall compared to the inner wall found by Leach (1980) and Leach and Zoerb (1983). In this way, these authors believed that the fewer tubules in the inner wall would minimise the effect of the medullary cavity acting as voids when the tissue is vertically loaded resulting in the inner wall being less rigid than the outer wall.

The tubule densities for the data sets displayed a *non*-normal distribution, similar to that found for both pony and horse hoof (Reilly *et al* 1996, 1998b). Consequently, the mean of the untransformed data of ~12 tubules mm^{-2} for the morbid capsules,

provided an overestimate for the tubule density compared to the mean which was determined from transformed data of ~10 tubules mm^{-2}. The results confirm the point made by Reilly *et al* (1996) and Reilly (1999) that the use of untransformed data can lead to an overestimation of tubule density.

Both Figure 5.2 and Figure 5.5 follow a similar trend with a decrease in tubule density in a dorso-palmar direction towards the middle of the hoof wall and then little change over the remainder of the hoof wall. However, Figure 5.2 shows a decrease in tubule density to ~40% HWD for clippings, whereas for morbid hoof, Figure 5.5 and Figure 5.6 show a decrease to ~50% HWD. This difference may indicate that part of the outer *Stratum medium* for the clippings was missing due to wear or rasping by the farrier.

The individual results for clippings and morbid samples will be discussed separately.

5.5.2 Clippings

The data from donkey hoof clippings showed a *non*-normal distribution and could not be transformed to produce a normal distribution. The protocol of Reilly *et al* (1996) could not therefore be used to establish parameters for fixing the boundaries for donkey hoof clippings. The boundaries previously used for pony hoof by Reilly *et al* (1996) were therefore used for comparative purposes only.

For clipping samples, zone 1 (0-26% HWD) showed a steep decline in tubule density from ~35 tubules mm^{-2} to ~10 tubules mm^{-2}. This tubule density within this zone was also significantly different from the remaining zones. Zone 2 (26-51% HWD) displayed a relatively constant tubule density of ~9 tubules mm^{-2}. This also existed for 51-77% HWD for zone 3. Although tubule density appeared relatively constant for zones 2, 3 and 4, there was actually a significantly higher tubule density in zone 4 when compared to zone 3. This therefore points to the *Stratum medium* being divided into three zones by tubule density.

5.5.3 Morbid Samples

For morbid samples, zone 1 (0-31% HWD) showed a steep decrease in tubule density from ~35 tubules mm^{-2} to ~15 tubules mm^{-2} with zone 2 (31-52 %HWD) displaying a slight decrease in tubule density from ~15 tubules mm^{-2} to ~10 tubules mm^{-2}. Zone 3 and 4, comprising the remainder of the hoof wall, showed a constant tubule density of ~9 tubules mm^{-2}. This differs from the comments of Hifny and Misk (1983) who described the tubules as being distributed in two layers (which is believed to mean two zones).

There was no significant difference between tubule density in zones 3 and 4 but there were significant differences between all other zones. This was also consistent with the zonal moisture content and hydrated regain results for these two zones. There was also no significant difference between the slopes of the regression lines.

This analysis therefore indicated that the *Stratum medium* of donkey hoof at 50% HWH has a three-zoned pattern of tubule density rather than the four-zoned pattern shown by horses and ponies.

5.5.4 Comparison Between Tubule Densities for Clipping and Morbid Samples

The overall tubule density for clippings was significantly lower than for samples taken at 50% HWH. The median tubule density for donkey hoof clippings for zone 1 and 2 was significantly lower than the calculated tubule density for zone 1 and 2 for morbid hoof (Table 5.2). This may be due to the influence of part of zone 1 being missing. However, there was no significant difference between the tubule density values for zones 3 and 4 for clippings and those for the morbid samples. Ideally, clippings and 50% HWH samples should be compared when removed from the same capsule to reduce the effect of possible differences being due to samples being used from different animals. Unfortunately, this was not possible at the time as the clipping area of the morbid capsules was damaged.

The use of boundaries at 26%, 51% and 77% HWD for the donkey hoof clippings resulted in zone 1 being 5% of HWD narrower than for the morbid samples and may provide one reason for the differing tubule densities in zone 1. The data set was therefore re-examined using the morbid donkey hoof boundaries to see whether this did, indeed, make an appreciable difference to the results. The results are shown in Table 5.3. It was evident, however, that this hardly changed the results and still resulted in the same differences for zone 1 and 2 between clipping and morbid samples.

Table 5.3 - Donkey Clipping Tubule Density Zones Adjusted According to Morbid Donkey Hoof Boundaries (* from Table 5.2)

	Non-adjusted Tubule Density*		Adjusted Tubule Density	
	Mean	**Median**	**Mean**	**Median**
All Zones	10	9	10	9
Zone 1	16	14	14	14
Zone 2	9	8	8	8
Zone 3	8	8	8	8
Zone 4	9	9	9	9

Again, the lack of difference may be due to the sample area of the clipping in unshod animals becoming damaged or worn. There was no significant difference between a statistical comparison of HWDs between clippings and samples taken at 50% HWH. However, the clipping samples did show a mean difference of 10% in the depth of the *Stratum medium* although this was not significantly different. This, together with the differences indicated between Figure 5.2, Figure 5.5 and Figure 5.6 indicated that the tubule density for zone 1 in clippings may not therefore be a true tubule density. However, the tubule densities found in zones 3 and 4 were consistent with the results found in morbid hoof. These factors should be taken into account when clipping samples form the basis of an experimental study.

Both clipping and morbid samples showed a different three zone pattern of tubule density. It is believed, however, that the loss of the outer *Stratum medium* for the clipping samples may have resulted in this difference. This possible influence must be taken into account in future work.

The following comparison with horse and pony hoof horn has therefore been carried out using the results from the morbid samples.

5.5.5 Comparison Between Tubule Densities for Donkey, Pony and Horse Hoof Horn

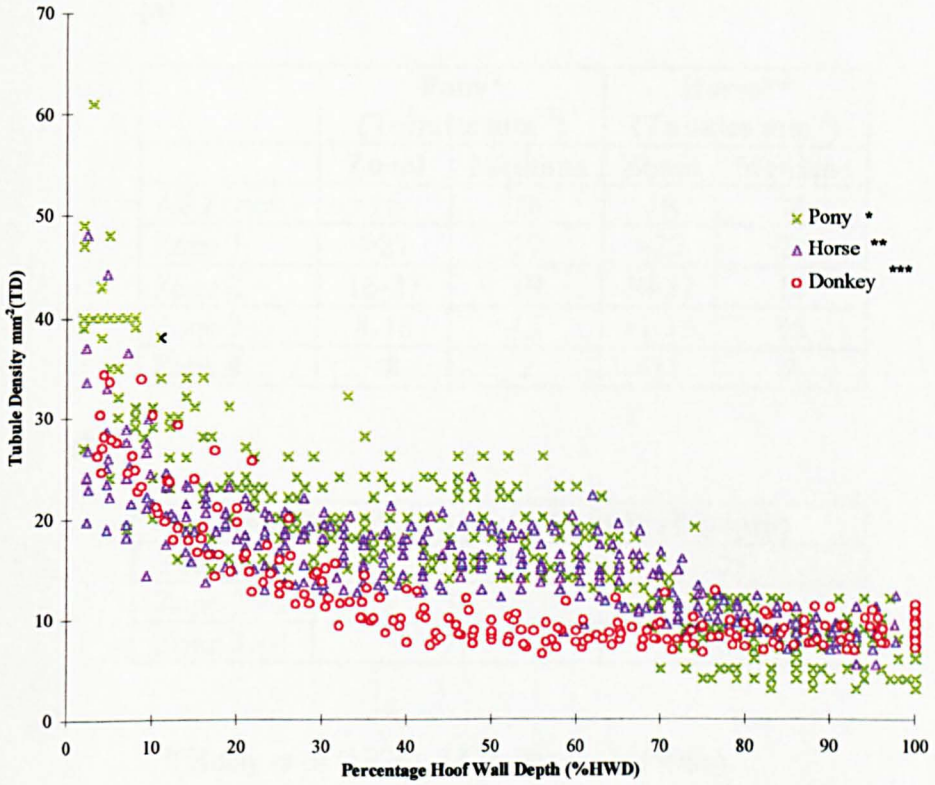
The plots of %HWD against tubule density in this thesis for morbid samples did not indicate a four zoned step-like pattern that has been observed for pony (Reilly *et al* 1996; Reilly 1999) and horse hoof (Reilly *et al* 1998b) but indicated that donkey hoof horn may be properly divided into three zones by tubule density rather than four zones. These results have provided quantitative confirmation of the belief of Tohara (1948), Hifny and Misk (1983) and Reilly (1997) that differences in the distribution of tubules exist between horse and donkey hoof.

A comparison was made between morbid donkey samples and pony and horse hoof. On the basis of this analysis, the median tubule density for donkey hoof at ~10 tubules mm^{-2} is lower than for horse or pony at ~16 tubules mm^{-2} (Table 5.4). The tubule density ranges listed in Table 5.2 for morbid donkey hoof in zone 2 were 16-34 mm^{-2}, whereas those for pony and horse hoof were previously found to be 16-27 mm^{-2} and 16-22 mm^{-2} respectively (Table 5.4).

The slightly greater depth of zone 1 for morbid donkey hoof of 31%, as opposed to 26% in pony hoof and 25% in horse hoof, may be a contributory factor. The tubule densities in zone 3 of donkey, pony and horse hoof were similar at 10-16, 8-16 and 11-16 mm^{-2} respectively. Pony tubule density in zone 4 of <8 mm^{-2} (Reilly *et al* 1996) was slightly less than that found for both horse (Reilly *et al* 1998b) and donkey hoof horn at <10 mm^{-2}. This can be seen graphically in Figure 5.8. However, median tubule densities for zones 2 and 3 for donkey hoof are lower than

those for horse or pony hoof. The difference in zone 3 is particularly clear in Figure 5.8 which also indicates that zone 4 is similar to horse hoof.

Figure 5.8 - Comparison of Tubule Density by Percentage Hoof Wall Depth at the Midline Dead Centre of the *Stratum medium* of Donkey, Horse and Pony Hoof



* from Reilly *et al* (1996)
** from Reilly *et al* (1998b)
*** from this study

This lower number of tubules in zone 3 for donkey hoof at 52-76% HWD may be linked to tubule size as tubule size in this area appears to be greater than that shown in horse hoof (Hopegood, L. personal observations). A reduced tubule density would follow an increase in tubule size as there would be less available space for the packing of tubules. Indeed, Warzecha (1993) believed there may be an association between the large diameter of the tubules and the smaller number of tubules in the

inner wall of hoof horn of horses. This was confirmed quantitatively by Reilly (1999) who found a significant negative correlation between tubule density and tubule size for zones 1-3 for pony hoof horn.

Table 5.4 - (a) Comparison of Zonal Tubule Densities and (b) Zonal Divisions of the *Stratum medium* at the Midline Dead Centre for Pony and Horse Hoof

(a)

	Pony* (Tubules mm^{-2})		Horse** (Tubules mm^{-2})	
	Zonal	**Medians**	**Zonal**	**Medians**
All Zones	16	16	16	16
Zone 1	>27	27	>22	21
Zone 2	16-27	19	16-22	16
Zone 3	8-16	15	11-16	15
Zone 4	<8	7	<11	9

(b)

Zonal Boundaries (as % Hoof Wall Depth)		
Zone 1 - 2	26	25
Zone 2 - 3	51	47
Zone 3 - 4	77	69

* Reilly *et al* (1996), ** Reilly *et al* (1998b).

A comparison of previously published zonal tubule density for horse hoof with donkey hoof zonal tubule density is shown in Table 5.5. Descriptions of sample sites and definitions of zones have often not been provided in detail. The early work of Chauveau (1853, according to Fleming 1871a) and Rössner (1940) examined an outer, middle and inner zone but a detailed description of the divisions of the HWD were not provided. However, their results were similar to those found for donkey hoof horn in the present study.

Bucher (1987) included the results from hind hooves which may influence the tubule density of hoof horn owing to the differences in hoof shape and angle between the

front and hind hooves. This may be a reason that the tubule density results for the outer and inner zones described by Bucher (1987) were lower than those found in this thesis.

Table 5.5 - Comparison of Previously Published Zonal Tubule Density for Horse Hoof with Donkey Zonal Tubule Density

Author	Description of Area	Tubule mm^{-2}	Differences to this Study	Comments
This study	Zone 1 Zone 2 Zone 3 Zone 4	>34 16-34 10-16 10	N/A	N/A
Chauveau (1853) (in Fleming 1871a)	Outer zone Middle zone Inner zone	25-30 15-25 8-12	} } similar }	Zonal descriptions not provided
Rössner (1940)	Outer zone Middle zone Inner zone	15 9 7	} } similar to median } zonal values	3 sample sites according to Tscherne (1910)
Bucher (1987)	Outer zone Inner zone	14 8	< zone 1 and 2 slightly lower than zone 4	Included results from hind hooves
Reilly *et al* (1996) and Reilly (1999)	Zone 1 Zone 2 Zone 3 Zone 4	>27 16-27 8-16 <8	Lower Smaller range, median values higher Similar but median values higher Similar	Pony hoof See text
Kasapi and Gosline (1997)	Outer wall Inner wall	25 10	< zone 1 similar to zone 3	6 sample sites used
Reilly *et al* (1998b)	Zone 1 Zone 2 Zone 3 Zone 4	>22 16-22 11-16 <11	Ditto Reilly *et al* (1996)	Horse hoof See text

Kasapi and Gosline (1987) examined six sample sites across the HWD of three horses but only reported the figures for an outer and inner wall. Their results for tubule density for the outer wall were less than that shown in this study but the results from the inner wall were similar to zone 3 in this thesis. There will always be the difficulty of the outer wall being influenced by rasping by the farrier and this, together with the possible species difference, may account for these differences.

5.5.6 Hoof Wall Depth

Factors that have been thought to influence tubule density include bodyweight, nutrition, age, season, breed, genetics and housing. The positive correlation shown between HWD and bodyweight (0.92) may mean that a mechanism exists whereby the area between the papillae is able to respond to the animal's bodyweight to produce the width of *Stratum medium* capable of protecting the contents of the hoof and supporting the animal's weight. There was no significant inverse relationship between HWD and tubule density contrary to the beliefs of Rössner (1940). Following the relationship established between bodyweight and HWD, it may have been expected to see a correlation between bodyweight and tubule density as has been shown for cattle hoof (Walz 1951) and for pony hoof (Reilly 1999). This relationship did not exist for bodyweight and tubule density from the full HWD for donkey hoof clippings. However, it did exist between bodyweight and tubule density for zone 3 for clippings. This relationship agreed with the findings of Reilly (1999) for zone 3 for pony hoof horn. Reilly (1999) also found a significant correlation between bodyweight and tubule density for zone 2. Although this was not seen for donkey hoof horn, the correlation was -0.551 with a p value of 0.051. It is interesting to note these similarities between donkey and pony hoof horn and the effect of bodyweight on tubule density in zone 3. This may be an area that is particularly stressed during movement of the animal. These results also indicate that the area of the coronary papillae responsible for the production of tubules for zone 3 may be particularly affected by bodyweight compared to the other zones.

There was unlikely to have been an effect caused by nutrition on tubule density as the animals were on similar diets which was a feature of The Donkey Sanctuary regime. Following biotin supplementation trials, Dittrich *et al* (1994) had reported an increase in tubule numbers and Reilly (1999) had found a change in tubule density in zone 4 for pony hoof horn following supplementation. A seasonal influence would not have been expected as the samples from clippings were taken within a one month period. It was not possible, however, to investigate the effect of breed,

genetics and housing on tubule density as the full history of the animals was not known.

5.5.7 General Discussion

Reilly *et al* (1996) stated that the finer functional properties of the hoof are likely to be dictated by contributions from different levels of its structural organisation. They believed that a variation in tubule density would confer differences in mechanical properties, regulated by moisture content, across the hoof wall. The functional significance of zonal tubule density has yet to be elucidated, although Kasapi and Gosline (1997) thought that the existence of a specific pattern of tubule density may reflect the variation of functional demands placed on different parts of the hoof. The arrangement of tubules distributed within the matrix of intertubular horn, with the tubules acting as a reinforcement for the less ordered material of the continuous phase, has been considered as being like that of a synthetic, unidirectional fibrous composite (Reilly *et al* 1996; Cope *et al* 1998; Newlyn *et al* 1998). If this analogy is valid, the mechanical properties, density and disposition of the tubules will all influence the physical properties of hoof horn. It is important to establish the 'normal' tubule density for healthy donkey hoof as this may provide data for comparison with hoof samples that have been altered pathologically.

The anatomical differences in shape and angle of hoof between the species may have resulted in the differences in zonal tubule density. In horses, movement of the anterior aspect of the hoof wall occurs during weight bearing (Lungwitz 1883, 1891) and, more recently, Leach (1980) referred to this as a "dorsoconcavity". The four zoned tubule density arrangement which may act as a quadrilaminar ply composite material shown for horse and pony hoof may result in more movement, therefore allowing this dorsoconcavity to occur. The donkey possesses a more upright foot compared to that of horse or pony and this may result in different movement of the capsule when it contacts the ground.

If hoof horn is considered as a unidirectional fibre composite then the three zoned arrangement for donkey hoof horn may be an arrangement which resists

deformation. This may result in a different movement of the donkey capsule at the toe and heel areas during weight bearing when compared to that of the horse hoof capsule. Newlyn *et al* (1998) simulated a computer model of the donkey capsule under static loading which resulted in an outward expansion of the heels and a dorsoconcavity at the proximal part of the MDC. However, displacement at the heel occurs more proximally than distally. Whereas, in comparison, lateral heel expansion for horse hoof has been shown to be greatest at the bearing border (Thomason *et al* 1992). These differences between the heel expansion for donkey and horse hoof may be as a result of the assumptions made in the computer model or from actual differences between the species. The heel height is generally lower in horses than donkeys. However, horse hoof is thickest at the toe and becomes thinner towards the quarters and heels (Emery *et al* 1977) which may aid heel expansion. This tapering of the *Stratum medium* does not occur in donkey hoof (Fowler 1995, Reilly 1997).

The overall shape of a horse hoof is much rounder than that of the U-shaped donkey hoof. This, together with the obvious difference in hoof size, causes the curvature of the *Stratum medium* of donkey hoof at the dorsal wall to be greater than that for horse hoof. This, again, may influence the differences shown in expansion of the hoof capsule.

It is believed that the pattern of tubule density that exists towards the quarters and heels in donkey hoof has not been reported in the literature nor has it been studied in this project. If the three zoned arrangement of tubule density, which has now been established for donkey hoof at the MDC, continues around the *non*-tapering donkey hoof wall to the quarters and heels, then this may confer upon the donkey hoof mechanical advantages over the four zoned arrangement of tubule density which is seen for horse and pony hooves.

The transitions between the zones may allow for controlled delamination of the wall by acting as a quadrilaminar ply (Reilly *et al* 1996) as a transition between two zones of differing morphology and physical properties may result in decreased cohesive

abilities (Bolliger 1991). This would then act as a "fail safe" mechanism in preventing damage from reaching sensitive tissues (Reilly *et al* 1996). This was endorsed by Zenker *et al* (1995) who found intercellular microcracks in the area of transition from the inner to the middle zone of the *Stratum medium*.

The lower mean tubule density of ~10 tubules mm^{-2} for donkey hoof horn in this study compared to the ~16 tubules mm^{-2} shown for pony and horse hoof horn (Reilly *et al* 1996, Reilly *et al* 1998b) may mean that tubule density is related to wear as donkeys generally do not need to wear shoes. Schummer *et al* (1981) believed this relationship may exist but did not provide supporting information. This would be an area for further investigation.

Figure 5.6 shows the tubule density from morbid hoof for individual animals. This indicates there are differences between individuals. In particular, Donkey 1 and Donkey 7 show a higher tubule density for a given HWD over the first 30% HWD. However, the trend of an overall decreasing tubule density to ~50% HWD and then a similar level for the remainder of the hoof wall is still shown. Identification of individuals that do not conform to the data set as a whole may indicate an underlying pathological hoof condition.

As tubule density is the culmination of tubules forming on the papillae from the coronary corium, presumably there must be differences in the number of papillae produced between species. The overall results for tubule density of donkey hoof horn were lower than those shown for pony hoof horn. However, the results for zonal tubule density for the *Stratum medium* of donkey hoof horn are of a similar order to those previously found for pony and horse hoof by Reilly *et al* (1996) and Reilly *et al* (1998b) respectively. However, subtle differences do exist, in particular the lower median tubule density shown for zone 2 and 3 for donkey hoof horn. These differences lead to the tubule density pattern for the *Stratum medium* of donkey hoof horn being a three zoned pattern compared to the four zoned pattern previously found for both pony (Reilly *et al* 1996) and horse hoof horn (Reilly *et al* 1998b).

The distribution of tubules within the hoof may be linked with other quantifiable factors such as moisture content and mechanical properties. As the *Stratum medium* can be divided into zones, it was decided to look at these factors for each zone. The inter-relationships between these factors is discussed in Chapter 8.

5.5.8 Future Work

The results from this thesis so far for both moisture content and tubule density indicate that donkey hoof horn should be divided into three zones. The mechanical properties of zonal samples are investigated in Chapter 7. Based on these results it must then be decided whether future work should be conducted on purely a three or four zoned basis.

There is a need to carry out work on morbid capsules from a known donkey population but, as discussed in Chapter 2, it is unlikely that a number of these will be available.

Reilly and Kempson (1992) defined good quality hoof horn as that which allows its full and proper function to be fulfilled. This "working" definition of hoof quality needs to be explained. This present method of examining tubule density may provide a means of doing this by quantifying the difference between good and poor quality hoof horn as a previous attempt had been inconclusive (Tscherne 1910, cited in Rössner 1940). However, initial identification of these two types of horn would need to be subjective as the examination of tubule density by a quantitative means would establish whether differences do exist between good and poor quality hoof horn.

One way in which this method of ascertaining tubule density could be improved is to use an overlay grid that follows the curve of the outer hoof wall (Reilly 1999), although following the curve of the inner hoof wall would, again, avoid the problem of interference to the hoof wall by the farrier or other external influence. This would then take into account the tubule population at the edge of the hoof wall together

with that near the *Stratum internum* that is missed by the present grid. This would also allow the remaining counts to follow the shape of the hoof wall. From a practical point of view, however, this may be difficult to achieve as the curve of the wall varies between animals. Individual grids would therefore need to be established.

A more detailed count may be achieved by enlarging the image to a size that would enable an overlay grid to be used that would allocate 1% HWD to each cell. In practice, however, this may prove difficult as, for example, an 8 mm HWD would have to be divided into 100 counting cells.

Manipulation of images may be made easier by using digitised images. Similar techniques can be used to identify the tubule density for other hooved animals.

Tubule density can be compared with tubule sizes as Reilly (1999) found a link between these two characteristics. This may then explain the differences in tubule density between pony or horse hoof and donkey hoof horn as differences in tubule sizes may alter the packing capabilities and therefore the distribution of tubules.

A direct comparison of clippings and 50% HWH samples from the same capsule would reduce the likelihood of errors being introduced owing to the use of different animals.

The effect of tubule density on the resistance of hoof horn to wear should be investigated.

5.6 Conclusions

- A quantitative method was used to assess the tubule density at the midline dead centre of the *Stratum medium* of both clippings and morbid samples of donkey hoof horn.

- A three-zoned pattern of tubule density is suggested for donkey hoof horn, which contrasts with the four-zoned pattern of tubule density which has been reported for both pony and horse hoof horn. It is proposed that the hoof wall may act as a trilaminar ply.

- Differences in tubule density across the *Stratum medium* exist between horse, pony and donkey hoof horn.

- The overall tubule density results for clipping samples were significantly lower than those for morbid samples. This was influenced by the tubule density results for zones 1 and 2. However, the tubule density results for zones 3 and 4 were not significantly different from morbid samples. Clippings should therefore be used with caution but have provided a very useful tool to establish protocols and provide preliminary data for donkey hoof horn.

- The mean hoof wall depth for clippings was ~7 mm whereas that for samples taken at 50% hoof wall height was ~8 mm.

- Although there was no significant difference between the hoof wall depth of clippings and 50% hoof wall height samples, clipping samples showed a loss of ~10% of hoof wall depth when compared to that of morbid samples.

- A strong relationship exists between hoof wall depth and bodyweight of the animal.

- There was a significant relationship between bodyweight and tubule density for zone 3 for clippings.

6. MECHANICAL TESTING OF DONKEY HOOF HORN

6.1 Introduction

As anatomical differences exist between donkey and horse hoof, it is likely that the response of donkey hoof horn to loading may be different from that of horse hoof. Although many types of mechanical test have been used to establish the mechanical properties of horse hoof horn, only limited work has been carried out on donkey hoof horn. This involved using strain gauges (Chang *et al* 1993) and computer modelling (Newlyn *et al* 1998). The limitations of using both these methods have been discussed in the literature review.

Methods of examining horse hoof horn *in vitro* have provided a better understanding of the mechanical properties of hoof horn. The examination of donkey hoof horn by 3 point bending will enable the mechanical properties of donkey hoof horn to be assessed and then compared to horse hoof horn. Three point bending was particularly chosen as the test method for this study as the hoof wall has been shown to undergo bending forces (*e.g.* Hood *et al* 1991).

The inverse relationship between moisture content and the mechanical properties of horse and pony hoof horn (Leach 1980; Douglas *et al* 1996; Reilly 1999) has not been reported for donkey hoof horn. The work carried out on horse hoof has used three levels of hydration, for example, dried at room temperature, fully hydrated and at an *in vivo* moisture content (Reilly 1999). A 3 point bending technique also allows a material to be tested below the yield point. This enabled the same sample to be re-tested, in this instance, at four different levels of hydration to ascertain the mechanical properties of donkey hoof horn. The protocols for sample collection, storage and methods of hydration were established in previous chapters.

The tubule density results in Chapter 5 and the results from this present chapter provided quantitative measurements for donkey hoof horn. The interrelationships between these parameters were investigated in Chapter 8.

6.2 Aims

- To assess the mechanical properties of donkey hoof horn using a 3 point bending technique.

- To assess the influence of moisture content on the mechanical properties of donkey hoof horn.

- To compare the results with those previously found for horse hoof.

- To use the results to identify the interrelationships between the moisture content, mechanical properties and tubule density of donkey hoof horn (Chapter 8).

6.3 Materials and Methods

6.3.1 Compliance Test

Samples were tested by 3 point bending on an Instron 4302[10] materials testing machine with a load cell of 100 N. Prior to testing of the samples, the compliance of the testing system was determined. Machine compliance is the "give" of the load frame, load cell and bending rig combined. A test was carried out using a rigid aluminium block with a stiffness that was much greater than that of the load system. A compliance file was automatically created by the Series IX[11] computer software and recorded the displacement and load values. The software then subtracts the displacement at preset load points from the results for the sample being tested.

6.3.2 Calculation of Shear

It was necessary to ascertain the contribution of shear forces to the 3 point bending of the donkey hoof samples. This was calculated using a rearrangement of Equation 7 and also Equation 8.

[10] Instron Ltd, High Wycombe, Bucks.
[11] Instron Ltd, High Wycombe, Bucks.

The shear deflection at the centre of a centrally loaded, simply supported beam of rectangular cross sectional area, can be calculated from Equation 7:

$$G = \frac{(1 + \nu) E}{2}$$

If $\nu = 0.4$ $\quad G = 0.7 E$

Then $\quad \delta_s = \frac{3WL}{10 \times 0.7 E bd}$

Shear Deflection $\quad \delta_s = \frac{0.429 WL}{Ebd}$

Key:
G = Modulus of rigidity (shear modulus)
E = Young's Modulus
ν = Poisson's ratio
b = breadth
d = depth
L = length of beam
W = central load

The bending deflection for a centrally loaded beam from Equation 8:-

$$\text{(bending deflection)} \quad \delta_b = \frac{WL^3}{48EI} = \frac{WL^3 12}{48Ebd^3} = \frac{WL^3}{4Ebd^3}$$

Using a rectangular cross-section: $\quad I = \frac{bd^3}{12}$

These expressions were evaluated with the following dimensions to determine the effects of shear deflection on the sample modulus.

$b = 8$ mm $\quad d = 2$ mm $\quad L = 24$ mm

6.3.3 Calculation of Strain Rate

The maximum strain rate was calculated from Equation 9 using a beam depth of 2 mm, a span of 24 mm and a crosshead speed of 2 mm min^{-1}.

6.3.4 Preparation and Testing of Donkey Hoof Horn

Samples of 30 mm in length and 2 mm in depth were taken from the MDC of fifteen donkey hoof clippings from the left fore limb. The specimen dimensions suggested

by the ASTM standards for bending of various materials were not achievable owing to the restricted size and shape of the original hoof samples. A recommendation for the size of specimen for testing plastic was that the span should be 165 times the thickness of the sample (ASTM E855 1994).

The samples tested were then used in section 5.3.1 for analysis of tubule density. The samples were prepared and milled as in section 5.3.1 and were wrapped in Parafilm and stored in a refrigerator at 4°C. The samples were referred to as beams.

The samples were mechanically tested under four conditions in the following order:

- at an *in vivo* moisture content;
- following drying over phosphorus pentoxide for ten days;
- following equilibration at 75% RH for ten days.
- fully hydrated by placement in distilled water for four days;

The environment of 75% RH was chosen as an intermediate level between dried and hydrated samples.

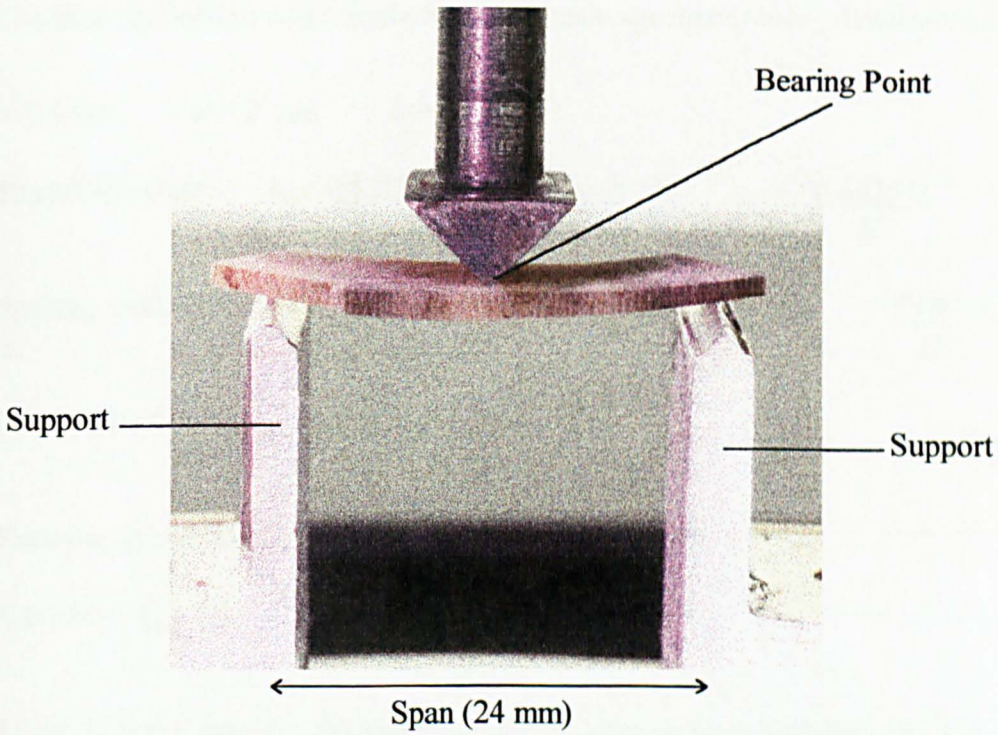
The moisture regain, hydrated regain and 75% RH hydrated regain were ascertained for comparative purposes prior to testing at the different levels of hydration. Sample dimensions of width (b) and depth (d) were taken prior to bending following equilibration of samples in their particular environment. Samples were immediately rewrapped in Parafilm following weighing.

Samples were removed from the environment and placed flat in the x plane and centrally across the two supports of the bending platform with a span of 24 mm with the most proximal part of the clipping uppermost and the bearing point centred over the sample (Figure 6.1). A span of 24 mm was chosen to provide a span to depth ratio of twelve. This also satisfied the recommendation of Jackson (1992) and Vincent (1992) to use a span to depth ratio of greater than ten. Samples were tested in the y plane to a deflection of 0.5 mm at a crosshead speed of 2 mm min^{-1}.

Samples were preloaded to approximately 0.04 N to minimise possible effects of backlash (ASTM E855 1994).

Each sample was tested three times consecutively. The time between bends was only to allow for pre-loading which only took a few seconds. Results were analysed from the second bend as the first occasionally showed settling on the supports, particularly when the samples were dry, and the third bend was of a confirmatory nature. Previous work (Hopegood, L. unpublished data) had indicated there was no significant difference between moduli of elasticity between the three bends.

Figure 6.1 - Hoof Sample Prepared for 3 Point Bending



6.3.5 Calculation of Modulus

The modulus (E) was obtained by the Series IX software from the slope of a least squares fit straight line made through the steepest linear region at the start of the curve and ended at the point where the test ceased. Tests were terminated prior to the yield point (ASTM E855 1994). The flexural modulus was determined using Equation 6.

6.4 **Results**

6.4.1 Shear

The shear deflection was calculated for the main specimens using dimensions of:

$b = 8$ mm $\quad d = 2$ mm $\quad L = 24$ mm

Shear Deflection $\quad \delta_s = \dfrac{0.429W \times 24}{E \times 8 \times 2} = \dfrac{10.296W}{16E} = \dfrac{0.6435W}{E}$

Bending Deflection $\quad \delta_b = \dfrac{W \times 24^3}{4E \times 8 \times 8} = \dfrac{13824W}{256E} = \dfrac{54W}{E}$

Total deflection $= \dfrac{54.6435W}{E}$

The error in considering shear bending only is given by:-

$$\% \text{ error} = \frac{(E_1 - E_2)}{E_1} \times 100$$

Where E_1 is the modulus derived assuming deflections from both bending and shear and E_2 is the modulus derived assuming deflection from bending only. This will result in an underestimate of E.

$$\% \text{ error} = \frac{54.6435 - 54}{54.6435} \times 100 = \frac{0.6435}{54.6435} \times 100$$

Therefore neglecting shear deflection will result in an underestimation of the modulus of:

$$\frac{0.6435}{54.6435} \times 100 = 1.18\%$$

This is considered to be acceptable.

6.4.2 Strain Rate

The strain rate was calculated by using Equation 9:

Therefore, using a crosshead speed of 2 mm min^{-1}, a span of 24 mm and a depth of beam of 2 mm:

$$Sr = \frac{V \times 6d}{L^2} = \frac{2 \times 6d}{L^2} = 0.042\ \varepsilon\ min^{-1}$$

or: $= \frac{0.042}{60} = 0.0007\ \varepsilon\ s^{-1}$ $= 700\ \mu\varepsilon\ s^{-1}$ or $0.7 \times 10^3\ \mu\varepsilon\ s^{-1}$

The maximum strain rate would therefore be $0.7 \times 10^3\ \mu\varepsilon\ s^{-1}$.

Key:
V = crosshead speed
L = span
Sr = strain rate
d = depth of beam

6.4.3 Donkey Hoof Horn

The force displacement curves showed a Hookean relationship. There were no significant differences between the results for E for the three different bends ($p>0.05$, Kruskal-Wallis test) although the slope of the plots increased slightly on successive cycles of loading. The modulus decreased with increasing hydration. A comparison of force displacement curves for one sample tested at different levels of hydration is shown in Figure 6.2.

Figure 6.2 - The Effect of Moisture Content on the Force Displacement Curves of Donkey Hoof Horn

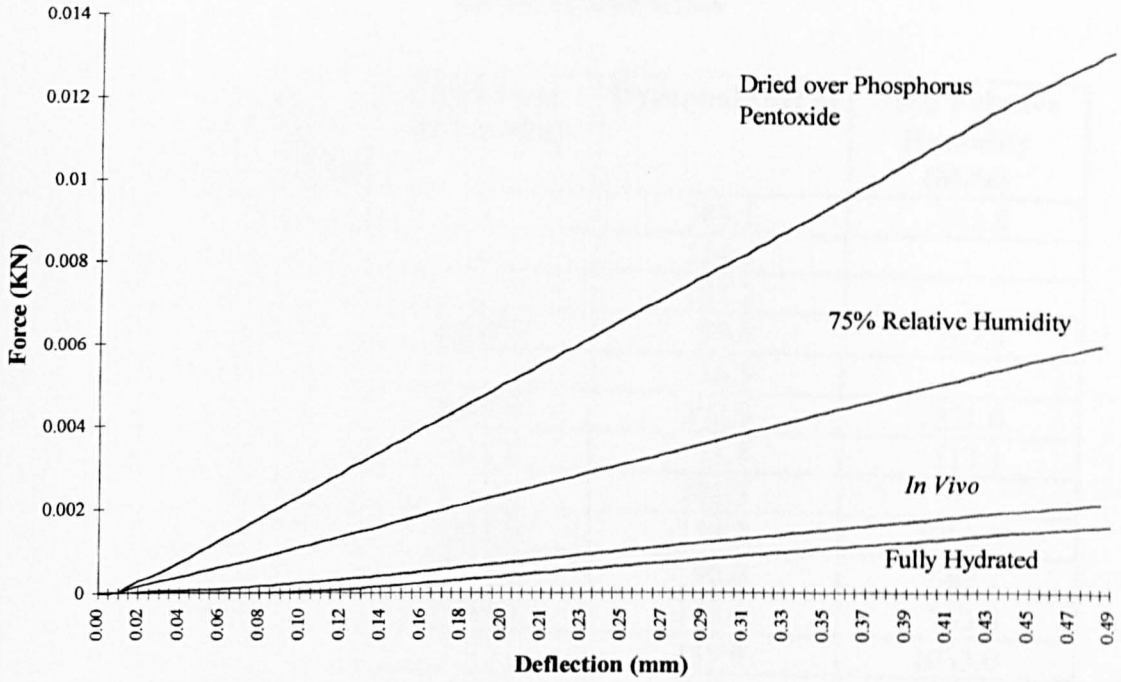


Table 6.1 shows the moduli results for individual samples at the different levels of hydration. There are no results for some samples as following drying over phosphorus pentoxide or equilibration in a 75% RH environment these samples were too curved and therefore too short to bend. The dimensions of *b* and *d* were generally reduced with a decrease in moisture content.

Table 6.1 - Modulus of Elasticity Results for Individual Samples Tested at Different Levels of Hydration

	In vivo Moisture Content (MPa)	Dried Over P_2O_5 (MPa)	Hydrated (MPa)	75% Relative Humidity (MPa)
	179.2	-	183.5	815.8
	111.5	-	114.5	-
	180.8	-	125.2	-
	169.3	1548.0	93.5	767.4
	207.8	-	136.5	-
	356.0	3189.0	202.9	1221.0
	129.1	1812.0	71.8	513.1
	224.3	-	201.3	-
	167.4	2381.0	158.2	1010.0
	137.8	2112.0	90.0	669.7
	210.8	1748.0	175.4	753.8
	174.8	-	137.9	1033.0
	175.1	-	127.9	854.6
	308.4	2371.0	172.9	963.8
	180.1	2180.0	188.4	781.1
Mean	187.4	2167.6	145.3	853.0
Median	177.2	1780.0	137.9	815.8
SD	67.6	509.9	41.9	194.3
CV (%)	36	24	29	23
p value	0.03	0.43	0.62	0.82

Key: SD Standard Deviation
CV Coefficient of Variation
P_2O_5 Phosphorus Pentoxide
- Represents samples that could not be tested

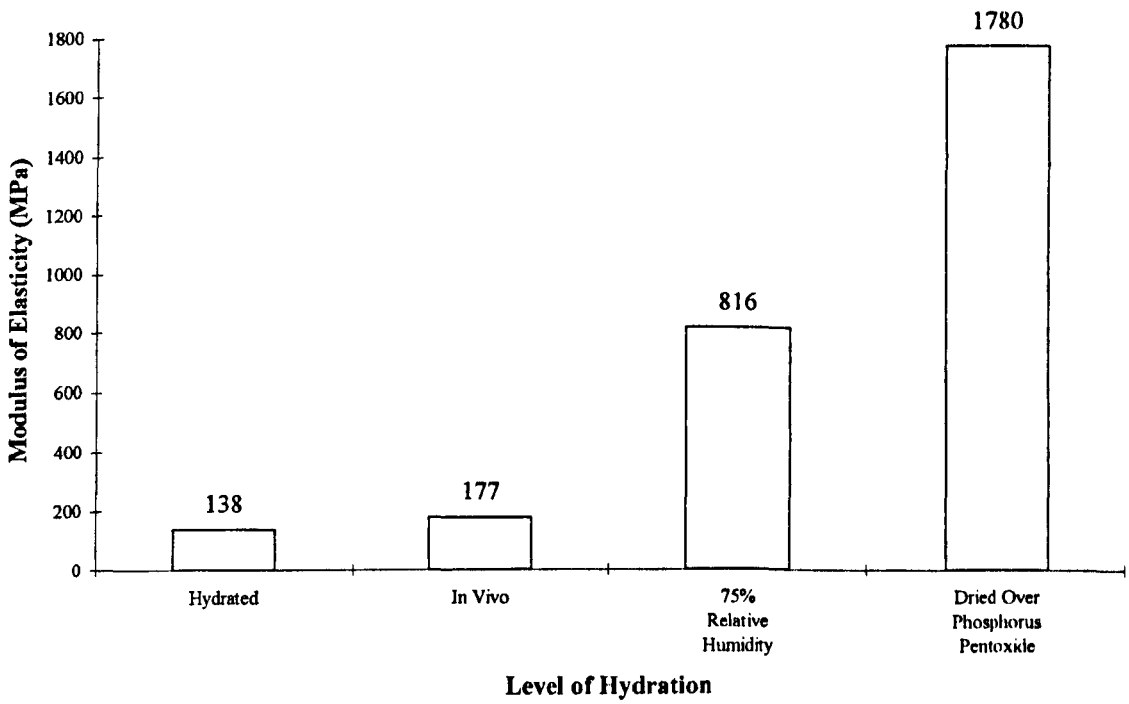
The normal probability plots for samples tested at fresh moisture content levels showed the data were *non*-normal ($p<0.05$). However, the results for samples tested fully hydrated, dried or at 75% RH showed normal distributions ($p>0.05$). Figure 6.3 shows a comparison of median moduli of elasticity for samples at different levels of hydration. There were significant differences between the moduli of samples tested at the different levels of hydration ($p<0.01$, Kruskal-Wallis). Mann-Whitney *U* tests indicated there were significant differences between all combinations except for

samples at an *in vivo* moisture content and those that were fully hydrated ($p>0.05$, Mann-Whitney *U* tests).

A Spearman correlation coefficient (r_s) demonstrated a significant negative correlation between moisture regain and modulus ($r_s = -0.85$, $p<0.001$). A linear regression analysis was performed of moisture regain against the modulus which was transformed by a log transformation. The result indicated a significant negative effect of water on modulus ($p<0.001$, $R^2 = 90\%$) as described by:

Log modulus = 3.21 - 0.0205 of moisture regain

Figure 6.3 - Comparison of Median Modulus of Elasticity for Donkey Hoof Horn Samples at Different Levels of Hydration



6.4.4 Moisture Content Results for Donkey Hoof Horn at Different Levels of Hydration

The results for moisture contents of the donkey hoof horn used for mechanical testing are shown in Table 6.2. Figure 6.4 shows the moduli of elasticity plotted against the moisture regain results for donkey hoof horn.

Table 6.2 - Moisture Content Results for Donkey Hoof Horn Samples Equilibrated at Various Levels of Hydration

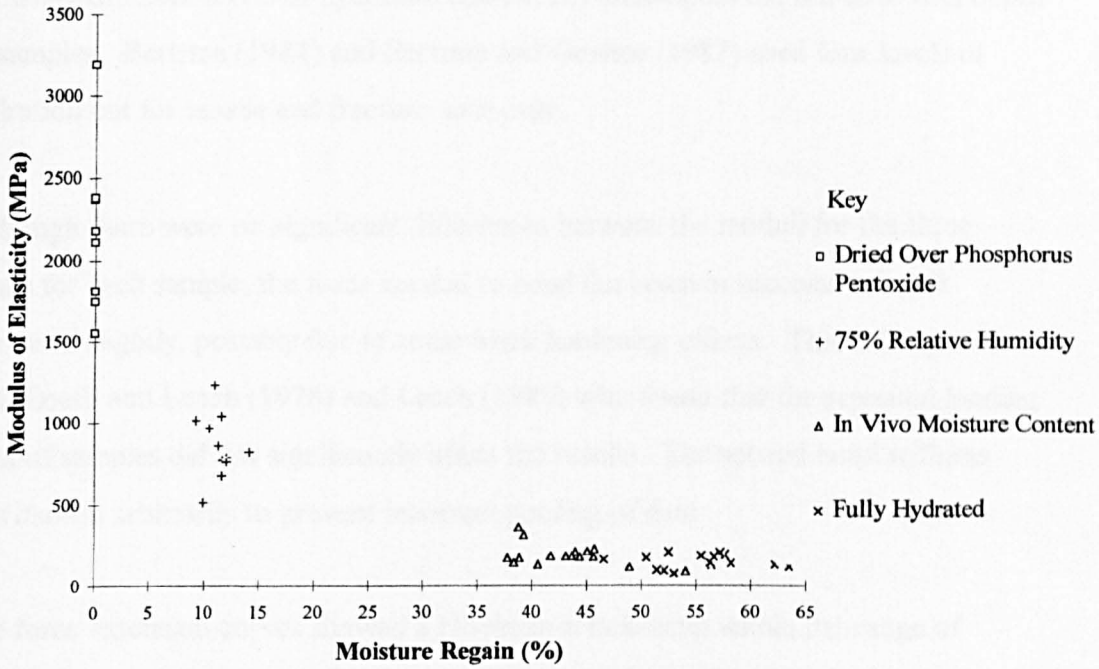
	Moisture Content (%)	Moisture Regain (%)	Hydrated Regain (%)	75% RH Hydrated Regain (%)
	29.42	41.69	55.49	14.08
	32.86	48.94	63.50	15.09
	35.08	54.04	70.82	13.35
	30.08	43.02	56.41	15.06
	30.53	43.96	58.22	11.78
	27.95	38.80	52.53	11.00
	28.86	40.58	53.01	9.93
	31.35	45.66	57.21	12.81
	27.98	38.85	46.56	9.28
	27.75	38.40	52.14	11.67
	31.06	45.06	56.65	12.04
	31.32	45.61	56.33	11.67
	30.73	44.36	62.19	11.35
	28.18	39.23	50.45	10.50
	30.38	43.64	57.65	12.09
Mean	30.24	43.46	56.61	12.11
Median	30.38	43.64	56.41	11.78
SD	2	4	6	2
CV (%)	7	10	10	10
p-value	0.34	0.15	0.28	0.36

Key: SD Standard Deviation
CV Coefficient of Variation
RH Relative Humidity

There was a significant difference between the median moisture contents for moisture regain, hydrated regain and 75% RH hydrated regain ($p<0.01$, Kruskal-

Wallis test). There was a significant difference between all combinations of moisture regain, hydrated regain and 75% RH hydrated regain ($p<0.01$, Mann-Whitney U tests).

Figure 6.4 - Moduli of Elasticity Plotted Against Moisture Regain for Donkey Hoof Horn



6.5 Discussion

6.5.1 General

This is the only reported study that provides results for the mechanical testing of donkey hoof horn. There has been only one previous study using 3 point bending on the full hoof wall depth of pony hoof (Reilly 1999).

This study is the only study that provides a comprehensive measure of the effect of four different levels of hydration on the mechanical properties of hoof horn in 3 point bending. Reilly (1999) carried out 3 point bending tests on pony hoof horn at three levels of hydration, namely fresh, fully hydrated and dry. His study was, however, following drying at room temperature and not following drying over phosphorus pentoxide for both mechanical testing of dry samples and for ascertaining sample moisture contents. Hinterhofer (1996) also carried out 3 point bending, but this was not using different levels of hydration and did not encompass the full hoof wall depth of samples. Bertram (1984) and Bertram and Gosline (1987) used four levels of hydration but for tensile and fracture tests only.

Although there were no significant differences between the moduli for the three bends for each sample, the force needed to bend the beam in successive bends increased slightly, possibly due to some work hardening effects. This is in agreement with Zoerb and Leach (1978) and Leach (1980) who found that the repeated loading of hoof samples did not significantly affect the results. The second bend stiffness was chosen arbitrarily to prevent incorrect pooling of data.

The force extension curves showed a Hookean relationship within the range of deflections used which agreed with previously published reports for horse and pony hoof horn (Zoerb and Leach 1978; Leach 1980; Landeau *et al* 1983; Reilly 1999).

Comparison of mechanical testing of hoof results is difficult as methodologies are often unclear. As has been explained, factors that may contribute to the variation in the reported modulus values include the type of mechanical test carried out, the region of hoof tested, hydration levels and the strain rate. These factors have all been taken into account in this study.

6.5.2 The Effects of Moisture Content on the Moduli of Hoof Horn

Any comparison of flexural moduli with other moduli calculated in compression or tension must be carried out with caution. Beams in flexural testing are subjected to

both tensile and compressive forces. The flexural modulus is therefore an approximate value of the true modulus (BSEN 2746 1998).

After controlling for moisture content by either drying or hydrating samples, the resultant data produced showed a normal distribution. This compared to a *non-*normal distribution of data from tests at *in vivo* moisture content with a high coefficient of variation indicating a high variability within the sample. It is therefore necessary that the moisture content of samples is brought to a reproducible level prior to mechanical testing. This was achieved by testing at specific levels of hydration. The results of Landeau *et al* (1983) also showed a large degree of variability in the modulus values. Coefficients of variation are generally not provided by other authors but it does provide a useful means of comparison between samples tested at different levels of hydration.

Many authors have previously recorded a decrease in modulus for horse hoof with an increase in hydration (Butler and Hintz 1977; Leach 1980; Bertram and Gosline 1986 and 1987; Küng 1991; Küng *et al* 1993; Douglas *et al* 1996; Hinterhofer 1996; Douglas 1998; Hinterhofer *et al* 1998; Reilly 1999). This was also the case for donkey hoof horn in this study. The significant negative correlation between moisture content and moduli found in the present study implies only that these vary together. However, the regression analysis indicated there was a relationship between modulus and moisture content, with an increase in moisture content resulting in a decrease in modulus. In fact, the effect of moisture content on the modulus of donkey hoof is the predominant factor as 90% of the variation in modulus was attributed to the effect of moisture content.

The extreme differences in moduli at different levels of hydration may be explained by the presence of differing amounts of water in the samples which would affect the amount of plasticisation of the sample. There would be considerable hydrogen bonding between protein polymers in the matrix for the dried samples, decreasing the mobility of the matrix and forcing it to become like a "rigid glass" (Fraser and MacRae 1980). Conversely, with fully hydrated samples there is a lack of secondary

bonding which results in greater movement and an increase in free space, both of which result in a reduced stiffness.

Samples equilibrated at 75% RH had a 4.5 fold decrease in hydrated regain over fully hydrated samples which resulted in a modulus that was six times higher than in fully hydrated samples. In bending, the modulus for dry pony hoof horn was four times higher than for hydrated samples (Reilly 1999). Dry donkey hoof samples possessed a modulus thirteen times greater than fully hydrated hoof horn. It must, however, be remembered that donkey hoof horn was dehydrated over phosphorus pentoxide which dried the hoof to a greater extent than the room temperature drying carried out for pony hoof horn. Dry horse hoof, which was not dried to as great an extent as donkey hoof samples, was thirty six times higher in tension than fully hydrated samples (Bertram 1984; Bertram and Gosline 1987). This much higher difference in modulus between dry and hydrated horse hoof may be as a result of the mechanical test involved. If, however, there is a true difference, this may indicate a species difference. This may mean, from a practical point of view, that as indigenous donkeys generally live in hot, dry climates, there would be less of an increase in hoof stiffness in very dry conditions than would possibly be seen for horse hoof. Drying of the whole capsule would result in shrinkage and increased pressure on the underlying structures. The above differences between donkey and horse hoof could indicate that drying of hoof samples has a much greater effect on the tensile modulus than on the flexural modulus. It may therefore be better for the hoof wall if it acted more by a combination of tension and compression, *i.e.* in bending, than in tension alone where there may be a risk of failure in dry conditions.

Bertram and Gosline (1987) suggested that, *in vivo*, horse hoof exists naturally at a moisture content in equilibrium with 75% RH. However, from these present results it can be seen that there are significant differences in moduli between samples tested at fresh moisture content and those stored at 75% RH ($p<0.01$, Mann-Whitney U test). Donkey hoof horn does not therefore exist at a moisture content received from a 75% RH but at nearly a fully hydrated moisture content.

There was a significantly higher hydrated regain of 56% compared with the moisture regain of 43% but this difference was not reflected in a significant difference in modulus. This may indicate that there is a maximum hydration beyond which there is no change in hoof stiffness. This may be a "fail safe" mechanism afforded by the hoof in particularly wet conditions, thus enabling the hoof to absorb deformation without total collapse of the structure. It may also be a mechanism to help avoid continual expansion or contraction of the hoof capsule due to changes in moisture content.

It is likely that somewhere between 75% RH and *in vivo* moisture content the modulus decreases. This may indicate the onset of plasticity and may be associated with the way in which water is bound in hoof horn.

The discussion will now focus on the results from the present study for donkey hoof horn and those of Reilly (1999) for pony hoof horn.

A direct comparison of results from this study with those of Reilly (1999) for pony hoof horn is shown in Table 6.3. The study involved the effects of supplementary dietary biotin on pony hoof horn. Results from his study have been combined for treatment and control groups for samples tested fresh and those tested dry as there were no significant differences between moduli or moisture regain ($p>0.05$, Mann-Whitney *U* test). The treatment group was those animals receiving supplementary biotin. Results for hydrated samples remain separate as there was a significant difference between treatment and control groups for moduli but not for moisture regain.

The results between donkey and pony hoof horn were of a similar order. However, there was a significant difference between the fresh moduli for both species ($p<0.01$, Mann-Whitney *U* test). A statistical comparison could not be made for hydrated moduli as the data sets were not provided by Reilly (1999). It should also be noted that the method for ascertaining moisture regain by Reilly (1999) was to dry samples at room temperature. The moisture regain results would therefore be lower than

would be expected if samples had been dried over phosphorus pentoxide. This may account for the significant difference between the moduli for pony and donkey hoof horn. The pony hoof horn samples were also morbid samples and were not clippings.

Table 6.3 - Comparison of Moduli and Moisture Content Results of Donkey Hoof Horn with Pony Hoof Horn Results After Reilly (1999)

a) Fresh Hoof Horn

Description	Mean (MPa)	Mean Moisture Regain (%)
Pony	487	30
Donkey	177	43

b) Hydrated Hoof Horn

Description	Mean (MPa)	Mean Hydrated Regain (%)
Pony - Control	345	34
Pony - Treatment	398	35
Donkey	138	56

The results of Kasapi and Gosline (1996) and Kasapi (1997) are also not directly comparable with those of this study as a dynamic bending technique was used.

The median moisture content of beam samples was 30% compared with the 33% found in Chapter 2 for donkey hoof horn. The 3% difference was attributed to moisture loss during cutting and sample preparation but was an actual 10% decrease in absolute moisture content. This loss of moisture, however, still did not result in a significant difference between the flexural modulus of *in vivo* and fully hydrated samples. There would, therefore, be no need to attempt to achieve an *in vivo* moisture content prior to further bending tests as fully hydrated samples would produce similar results. Indeed, Bendit and Feughelman (1968) suggested that as biosynthesis of keratinous fibres occurred in an aqueous environment, the intermediate filaments and the matrix would be in mechanical equilibrium in a fully hydrated state and this appears likely to also be the case for donkey hoof horn.

A problem occurred with some clipping samples becoming warped following drying in a 75% RH environment and also following drying over phosphorus pentoxide. The samples generally curved in the direction of the arc of the sample. They could not therefore be subjected to bending tests. With timber it is necessary to control the rate of evaporation to the rate at which moisture is reaching the surface to help minimise the development of stresses (Pratt 1986). For hoof samples this may be achieved by starting to dry samples at a higher humidity before then subjecting them to drying over phosphorus pentoxide. Drying of samples immediately over phosphorus pentoxide and then testing them mechanically may not therefore be the most appropriate way even if it does offer control of moisture content.

Only Zenker *et al* (1995) and Ley *et al* (1998) are reported to have carried out materials testing on horse hoof clippings. Zenker *et al* (1995) reported a tensile strength range of 40.2-324 MPa, the upper end of which is similar to some of the results for fresh samples in this study. Their samples were kept at 65% RH which would actually have a considerable drying effect on the samples. A higher modulus would therefore have been expected. Ley *et al* (1998) found a tensile strength of 22-35 MPa which was lower than the modulus found for donkey hoof horn.

6.5.3 Strain Rate

Although the strain rate used in this study of 0.7×10^3 $\mu\varepsilon\ s^{-1}$ appeared low compared to other published work, the *in vivo* strain rate, which must vary enormously during locomotion, has not yet been reported. Following calculation of the strain rate using Reilly's (1999) data, the same strain rate had actually been used. The effect of a change in strain rate from 1.6×10^3 $\mu\varepsilon\ s^{-1}$ to 32×10^3 $\mu\varepsilon\ s^{-1}$, *i.e.* a factor of twenty, only increased the modulus by 14% (Kasapi and Gosline 1996; Kasapi 1997). Their results also indicated a fifty fold increase in strain rate resulted in a three fold increase in modulus. They also commented that the effect of strain rate was not quite as expected and was not nearly as great as the effect of hydration. The present work was therefore justified in concentrating on carrying out a comparison at a constant strain rate as the effect of a change in strain rate on the mechanical

properties of donkey hoof horn has not been assessed. It does, appear, however, that the effects of strain rate are unlikely to be a great source of error when compared with the effects of hydration.

6.5.4 Span to Depth Ratio

The span to depth ratio of twelve resulted in a modulus which is assessed to be only underestimated by 1.18% due to shear deflection. Whereas for the work carried out by Kasapi and Gosline (1996) and Kasapi (1997) for a ratio of seven, using Equation 7, shear deflection would introduce an error of 4% in the modulus.

6.5.5 Anisotropy

Despite the anisotropy that is thought to exist with regard to the mechanical properties of hoof horn, the values represent a consistent characterisation of the modulus of donkey hoof horn under the conditions outlined.

The effect of the natural curvature of the beams in the plane of bending has been investigated (Newlyn, H. unpublished data) and indicates that a radius of curvature of the specimen of 25 mm could result in an underestimate of the modulus by approximately 6%. The actual radius of curvature is likely to be higher so this result would be decreased. Despite these facts, the moduli results for this study also fall within results previously reported for horse hoof. It is probable that no assessment of mechanical properties in biological materials could fulfill all the necessary criteria for mechanical testing. The consistent sampling techniques used in this study have reduced further the possibility of errors due to sample position and technique.

Other techniques applied to the analysis of engineering materials are used to assess the properties of materials when used in a structure. In this instance, the structure has already been built. It is very difficult to isolate the material from the structural properties as sections removed from the hoof wall cannot represent the total properties of the hoof. However, it is known that the hoof can cope with various everyday insults but how it does this is still relatively unknown. Work on the

mechanical properties of hoof horn using an engineering approach will enable the structure to be related more easily to its function.

This study has identified the mechanical testing of donkey hoof horn by 3 point bending at four different levels of hydration. Chapter 7 investigates the mechanical testing of partial hoof wall depth samples.

6.5.6 Future Work

This quantitative analysis could be used in the future to compare samples from, for example, morbid hoof horn and clippings or even diseased donkey hoof horn to assess whether the hoof is compromised and has differing mechanical properties. Tests following a supplementary dietary trial to assess the effects of a feed supplement on the mechanical properties of hoof horn would also be useful.

The results from the present study on donkey hoof horn can now be input into the computer model of donkey hoof (Newlyn *et al* 1998) as moduli results were previously used from horse hoof.

Donkey hoof horn should be tested both in compression and tension and through different planes to see if it is, indeed, anisotropic.

Mechanical and hydration tests should be carried out above 75% RH to ascertain the moisture content necessary to result in a significant difference in modulus to the *in vivo* moisture content.

A study should be carried out to ascertain the effect of the alignment of IFs across the HWD on the mechanical properties of donkey hoof horn.

The same techniques used here should be used for horse hoof horn to ascertain whether there are real differences between the species.

6.6 Conclusions

- Donkey hoof horn has been shown to display a Hookean response during flexural testing by 3 point bending.

- The flexural moduli were similar to that previously shown for pony hoof horn.

- An increase in moisture content resulted in a decrease in modulus.

- The modulus increased 13 fold from hydrated samples to dried samples.

- There was a significant negative correlation between moisture content and modulus. Indeed, moisture content accounted for 90% of the variation in modulus.

- There was no significant difference between the modulus of *in vivo* samples and fully hydrated samples. This may indicate there is a level of hydration beyond which there is no further change in hoof stiffness, thus providing a "fail safe" mechanism.

7. ZONAL MECHANICAL TESTING

7.1 Introduction

A gradient of stiffness is believed to exist across the HWD for horse hoof horn (Leach 1980; Leach and Zoerb 1983; Küng 1991; Zenker *et al* 1995; Douglas *et al* 1996; Kasapi and Gosline 1997; Douglas 1998; Reilly 1999; Wagner *et al* 2001) which is believed to transfer the forces across from a stiff outer wall to a relatively soft inner wall (Kasapi and Gosline 1997). It is likely that many of the mechanical testing results for different parts of the HWD for horse hoof are as a result of the influence of moisture content as opposed to the mechanical properties of the material alone. The diverse tests carried out on different parts of the HWD for horse hoof horn were shown in Table 1.9 in the literature review although the only authors to have carried out 3 point bending on partial HWD samples were Hinterhofer (1996), Hinterhofer *et al* (1998) and Wagner *et al* (2001).

The mechanical properties of the different regions of the hoof wall depth of donkey hoof horn, together with the influence of moisture content on these properties, have not been investigated previously.

The differences in zonal tubule density shown for donkey hoof horn indicate that there may be differences in the mechanical properties across the HWD as tubule density may influence the mechanical properties of hoof horn (Chapter 5). Indeed, an investigation by Reilly (1999) identified the influence of tubule density on the bending stiffness of "fresh" hoof horn. The description "fresh" means that no alteration had been made to the moisture content of the samples following removal from the animal. The results indicated a negative correlation (-0.76, $p=0.029$) between mean tubule density in zone 3 and bending stiffness for the full HWD for pony hoof horn. An improvement on this protocol would be to compare tubule density for the individual zones with bending stiffness for individual zones. This present project has therefore identified a method to examine the influence of tubule density on the flexural stiffness of zonal samples.

Moisture contents have also been shown to differ across the different zones of the HWD of donkey hoof horn (Chapter 3). It is likely therefore that the resultant moduli for each zone will also be different. As for full HWD samples, there is likely to be an inverse relationship between mechanical properties and moisture content for zonal areas of the HWD. This has also been indicated for horse hoof horn (Leach 1980; Bertram and Gosline 1987; Küng 1991; Douglas *et al* 1996; Hinterhofer 1996; Hinterhofer *et al* 1998).

It would be useful to assess solely the mechanical properties of the zones by removing the effect of moisture content on the different zones. This will be assessed by the dehydration of samples and also by hydrating each zone to the same level of hydration.

The zones used for the mechanical part of this study for clippings are arbitrary as it had not proved possible to ascertain zonal boundaries by examining the tubule density of the clippings. Zonal boundaries were therefore defined as 25%, 50% and 75% HWD.

7.2 Aims

- To use 3 point bending to assess the mechanical properties of donkey hoof horn across the hoof wall depth.

- To assess the influence of moisture content on the mechanical properties of donkey hoof horn.

- To assess whether the mechanical properties across the hoof wall depth are solely related to moisture content.

7.3 Materials and Methods

7.3.1 Mechanical Testing of Zonal Donkey Hoof Clippings

7.3.1.1 Span, Strain Rate and Crosshead Speed

A span of 10 mm was used for zonal bending as preliminary work with zonal samples at a span of 24 mm resulted in the samples swinging on the supports. The sample depth was reduced to 1 mm in order to maintain a minimum span to depth ratio of ten. It was decided to maintain the same strain rate of 700 $\mu\varepsilon\ s^{-1}$ (0.042 $\varepsilon\ min^{-1}$) that was used for full HWD samples. The new crosshead speed to maintain this strain rate was then calculated from a rearrangement of Equation 9 from Chapter 1.

$$V = \frac{Sr \times L^2}{6d}$$ NB: the unit of the Sr used was $\varepsilon\ min^{-1}$

$$V = \frac{0.042 \times 10^2}{6} = 0.7\ mm\ min^{-1}$$

The crosshead speed needed to maintain the strain rate of 700 $\mu\varepsilon\ s^{-1}$ was therefore 0.7 mm min^{-1}.

7.3.1.2 Compliance Test

A compliance test was also carried out to determine the compliance of the entire testing system at this new 10 mm span (Chapter 6).

7.3.1.3 Calculation of Shear

Again, it was necessary to ascertain the contribution of shear forces to the 3 point bending of donkey hoof horn samples.

The shear deflections expected for the samples with a 10 mm span, 1 mm depth and 2 mm breadth were calculated using a rearrangement of Equation 7 and also Equation 8 from Chapter 1.

7.3.1.4 Preparation of Donkey Hoof Clippings

Samples were used from clippings from the midline dead centre of the left fore limb of five donkeys (Appendix 1). No further samples were available at this time as some of the donkeys in the sample population had developed foot problems. Samples were cut to 15 mm in length and milled to 1 mm depth according to the protocol for full hoof wall depth samples given in Chapter 5. Again, the loss of the external 10% of samples was taken into account as previously indicated for zonal moisture contents (Chapter 3). Zonal measurements at 25%, 50% and 75% hoof wall depth were marked onto the beams using a fine pen. As the samples were narrow, a scalpel was used to divide the wall into the four beams.

7.3.1.5 Testing of Donkey Hoof Clippings

Samples were then tested three times as before (Chapter 6) by 3 point bending at a crosshead speed of 0.7 mm min^{-1}. The results were, again, taken from the second testing of the beam. Samples were tested at the following moisture contents:

- *in vivo* moisture content;
- following full hydration;
- at 38% hydrated regain and
- following drying at room temperature.

Samples were dried at room temperature as they showed considerable warping when dried over phosphorus pentoxide. The *in vivo* moisture content samples and fully hydrated samples were prepared as in Chapter 6.

The hydrated regain previously found for zones 1-4 was 38%, 57%, 69% and 75% respectively (Chapter 3). One aim of this part of the study was to bring all the zones

to the same hydrated regain in order to attempt to eliminate any effects of different moisture contents across the HWD on the mechanical properties of the zones. As the samples were fully hydrated it would not have been possible to reproduce an hydrated regain of 75% in zone 1 as the maximum hydrated regain had been shown to be 38% for zone 1. It was therefore decided to reproduce an hydrated regain of 38% for all four zonal beams. The expected mass of samples at this level of hydration was calculated from the following:

Mass at 38% hydrated regain = Hydrated mass / (hydrated regain / 100) + 1) x 1.38

Zone 1 samples were placed in distilled water for four days and were then used for testing. Samples from zones 2-4 were also placed in distilled water until fully hydrated for four days. However, they were then removed and dried in air and weighed until they reached the calculated dry mass and therefore the equivalent of 38% hydrated regain. Samples were stored in Parafilm for four days prior to testing to allow for possible equilibration of moisture content across the sample.

The room temperature dried samples were left to dry for ten days at approximately 23°C.

The results of the moduli for the beams from the full HWD donkey hoof clippings were compared to a combination of the mean results for the combined moduli of zones 1-4.

7.4 **Results**

7.4.1 Calculation of Shear

$$G = \frac{(1 + \nu) E}{2}$$

If $\nu = 0.4$ $\quad G = 0.7 E$

Then $\delta_s = \frac{3WL}{10 \times 0.7\, E\, bd}$

Shear Deflection $\delta_s = \frac{0.429\, WL}{Ebd}$

Key:
G = Modulus of rigidity (shear modulus)
E = Young's Modulus
ν = Poisson's ratio
b = breadth
d = depth
L = length of beam
W = central load

$$\delta_s = \frac{0.429W \times 10}{E \times 2 \times 1} = \frac{2.145W}{E}$$
(shear deflection)

The bending deflection for a centrally loaded beam from Equation 8:-

$$\delta_b = \frac{WL^3}{48EI} = \frac{WL^3\, 12}{48Ebd^3} = \frac{WL^3}{4Ebd^3}$$
(bending deflection)

Using a rectangular cross-section: $I = \frac{bd^3}{12}$

$$\delta_b = \frac{W \times 10^3}{4E \times 2 \times 1} = \frac{125W}{E} = \frac{16.6975W}{E}$$
(bending deflection)

The error in assuming all deflection is due to bending only is given by:-

$$\% \text{ error} = \frac{(E_1 - E_2)}{E_1} \times 100$$

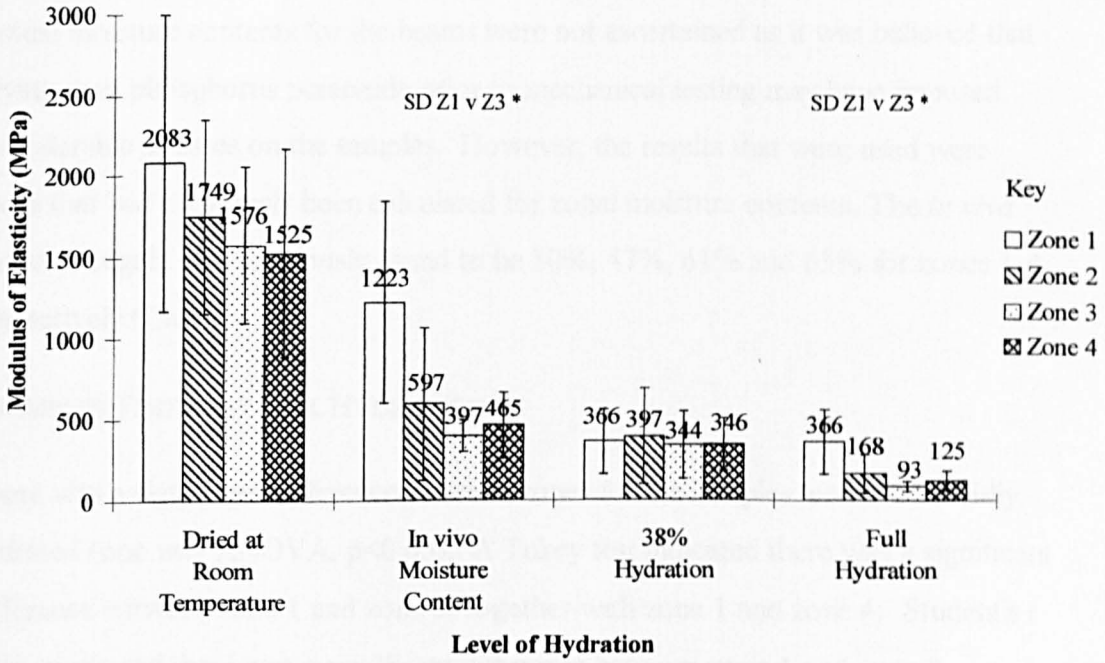
Where E_1 is the modulus derived from both bending and shear and E_2 is the modulus derived from bending only. This will result in an underestimate of E. Shear deflection therefore introduces an error of:

$$\frac{2.145}{125} \times 100 = 1.716\%$$

7.4.2 Donkey Hoof Clippings

The flexural moduli data for the different levels of hydration were normally distributed ($p>0.05$). The mean moduli of elasticity for bend 2 at the different levels of hydration for zonal bending are shown in Figure 7.1. The force extension curves showed a Hookean relationship as previously shown in Chapter 6 for full HWD samples.

Figure 7.1 - Mean Moduli of Elasticity for Bend 2 at Different Levels of Hydration for Zonal Bending



Key: SD Significant difference
* Significant difference $p<0.05$ by Student's *t* test
Error Bars Standard deviation bars

7.4.2.1 SAMPLES TESTED AT *IN VIVO* MOISTURE CONTENT

There was a significant difference between the flexural moduli for the different zones for the samples tested at an *in vivo* moisture content ($p<0.05$, one way ANOVA). A Tukey test indicated there was a significant difference between zone 1 and zone 3, together with zone 1 and zone 4. A student's *t* test confirmed there was a significant difference between zone 1 and zone 3 ($p<0.05$) but that there was no significant difference between zone 1 and zone 4. The coefficients of variation were very high at 51%, 79%, 24% and 44% for zones 1-4 respectively.

Actual moisture contents for the beams were not ascertained as it was believed that drying over phosphorus pentoxide prior to mechanical testing may have imposed considerable stresses on the samples. However, the results that were used were those that had previously been calculated for zonal moisture contents. The *in vivo* moisture regain was previously found to be 30%, 47%, 61% and 63% for zones 1-4 respectively (Chapter 3).

7.4.2.2 SAMPLES TESTED AT FULL HYDRATION

There was a significant difference between zones for the samples tested when fully hydrated (one way ANOVA, $p<0.05$). A Tukey test indicated there was a significant difference between zone 1 and zone 3, together with zone 1 and zone 4. Student's *t* tests confirmed there was a significant difference between zone 1 and zone 3 ($p<0.05$) but that there was no significant difference between zone 1 and zone 4. The coefficients of variation were 71%, 33%, 48% and 55% for zones 1-4 respectively.

The hydrated regains were as those calculated previously for clippings, *i.e.* 38%, 57%, 69% and 75% for zones 1-4 respectively (Chapter 3).

7.4.2.3 SAMPLES TESTED AT 38% HYDRATED REGAIN

There was no significant difference between zones for those samples tested at 38% regain (one way ANOVA, $p>0.05$). The coefficients of variation were 55%, 74%, 61%, 47% for zones 1-4 respectively.

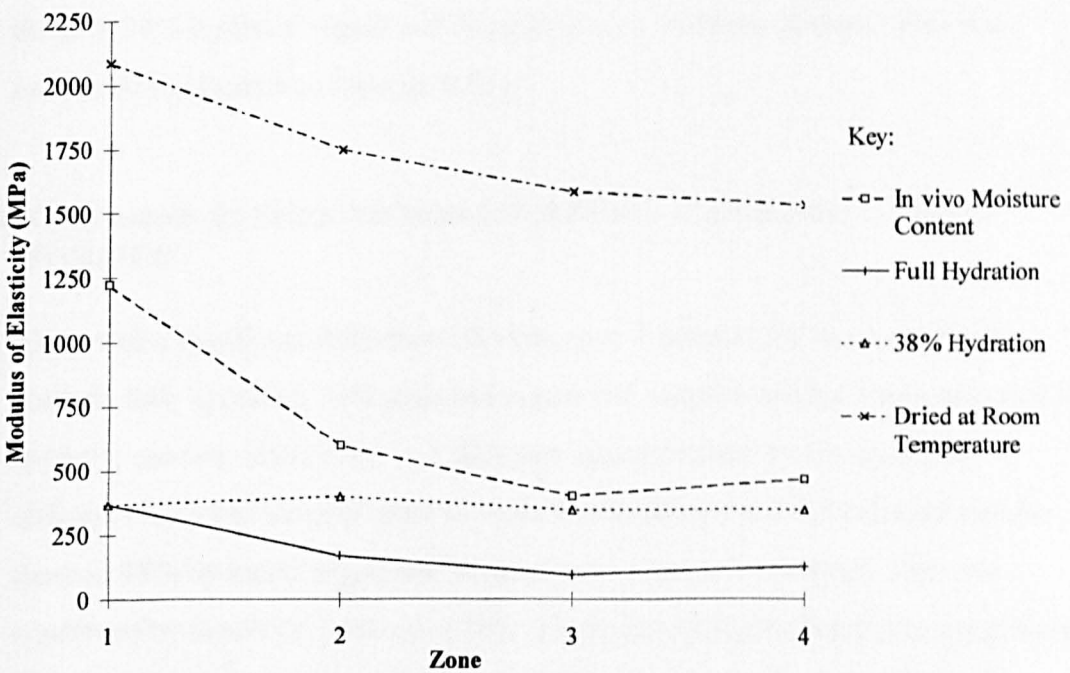
7.4.2.4 SAMPLES TESTED FOLLOWING DRYING AT ROOM TEMPERATURE

There was no significant difference between zones for those samples tested following drying at room temperature (one way ANOVA, $p>0.05$) although the mean moduli indicated a trend to decreasing moduli in a dorso-palmar direction. The coefficients of variation were 44%, 34%, 30%, 42% for zones 1-4 respectively.

7.4.2.5 COMPARISON BETWEEN MODULI FOR SAMPLES TESTED AT THE DIFFERENT LEVELS OF HYDRATION

Mean moduli for zonal samples tested at different levels of hydration are also shown in Figure 7.2 for ease of reference.

Figure 7.2 - Mean Moduli of Elasticity at Different Levels of Hydration



N.B. Points are joined for ease of reference only.

7.4.2.6 Comparison Between the Moduli for Zone 1 at Different Levels of Hydration

There was a significant difference between zone 1 samples for *in vivo* moisture content, fully hydrated, 38% hydrated regain and samples dried at room temperature ($p<0.01$, one way ANOVA). A Tukey test indicated there was a significant difference between moduli of samples dried at room temperature and those that were fully hydrated. There was also a difference between samples dried at room temperature and those at 38% hydrated regain. In addition to the above, Student's *t* tests indicated there was also a significant difference between the moduli of *in vivo* moisture content samples and those that were fully hydrated and at 38% hydrated regain ($p<0.05$).

7.4.2.7 Comparison Between the Moduli for Zone 2 at Different Levels of Hydration

There was a significant difference between zone 2 samples for *in vivo* moisture content, fully hydrated, 38% hydrated regain and samples dried at room temperature ($p<0.01$, one way ANOVA). A Tukey test indicated there were significant differences between samples dried at room temperature and fully hydrated samples, those at 38% hydrated regain and those at *in vivo* moisture content. This was confirmed by Student's *t* tests ($p<0.01$).

7.4.2.8 Comparison Between the Moduli for Zone 3 at Different Levels of Hydration

There was a significant difference between zone 3 samples for *in vivo* moisture content, fully hydrated, 38% hydrated regain and samples dried at room temperature ($p<0.01$, one way ANOVA). A Tukey test indicated there was a significant difference between samples dried at room temperature and fully hydrated samples, those at 38% hydrated regain and those at *in vivo* moisture content. This was confirmed by Student's *t* tests ($p<0.05$). These also indicated there was a significant difference between *in vivo* moisture content samples and fully hydrated samples ($p<0.05$).

7.4.2.9 Comparison Between the Moduli for Zone 4 at Different Levels of Hydration

There was a significant difference between zone 4 samples for *in vivo* moisture content, fully hydrated, 38% hydrated regain and samples dried at room temperature (one way ANOVA, $p<0.01$). A Tukey test indicated there was a significant difference between samples dried at room temperature and fully hydrated samples, those at 38% hydrated regain and those at *in vivo* moisture content. This was confirmed by Student's *t* tests ($p<0.05$). There were also significant differences between *in vivo* moisture content samples and fully hydrated samples and those equilibrated at 38% HMC_D and fully hydrated samples ($p<0.05$).

7.4.2.10 Comparison of the Moduli of Zonal and Full Hoof Wall Depth Beams

The moduli results for full HWD beams and the mean results for zonal beams are shown in Table 7.1.

Table 7.1 - Comparison of Zonal Moduli of Elasticity with Those for Full Hoof Wall Depth

Level of Hydration	***E* - Full HWD (MPa)**	***E* - Mean of Results of Zonal Beams (MPa)**
In vivo moisture content	194	670
Full Hydration	145	188
Dried	2168 (dried over phosphorus pentoxide)	1733 (dried at room temperature)

7.5 Discussion

There are many hoof studies involving the mechanical testing of horse hoof that encompass parts of the HWD but none encompasses the division of the wall into four zones. Mechanical testing of donkey hoof horn has not been previously reported. The results should be interpreted with caution owing to the limited number of samples

available, together with the resultant high coefficients of variation. It should also be borne in mind that zone 1 samples were not full width zones owing to the expected 10% loss of HWD.

This study provided a measure of the effect of four different levels of hydration on the mechanical properties of zonal beams of donkey hoof horn subjected to 3 point bending.

The force extension curves showed a Hookean relationship within the range of deflections used which agreed with previously published reports for horse (Leach 1980; Leach and Zoerb 1983) and pony hoof horn (Reilly 1999) and was similar to that for full HWD samples of donkey hoof horn.

Again, as for full HWD samples, and as was expected for zonal samples, the moduli were only underestimated by a small amount, namely 1.7%, because of the effects of shear.

In order to take into account the fact that samples were tested at an *in vivo* mass and this, together with the milling process, may cause differing results in flexural stiffness, samples were then placed in distilled water for four days and tested fully hydrated. The 38% hydrated regain was chosen as a level of hydration for all zones as this was the value for zone 1 when fully hydrated. This meant that samples from the other zones were then being tested at a similar moisture content to see if there were indeed differences in mechanical properties across the hoof wall depth which were not brought about by differences in moisture content. It was assumed that the moisture absorbed by samples which had been hydrated to 38% hydrated regain and had been stored in Parafilm for four days had equilibrated within the samples.

As was found previously (Chapter 6) for full hoof wall depth beams, the modulus of samples increased with decreasing moisture content (Figure 7.1) as has been found previously for horse hoof (*e.g.* Butler and Hintz 1977; Douglas *et al* 1996; Reilly 1999). For samples tested following drying at room temperature, at *in vivo* moisture

content and full hydration, the moduli generally decreased in a dorso-palmar direction. This mechanism may afford the contents of the hoof a protective gradient of stiffness by these levels of hydration and contribute towards the transfer of stress across the HWD.

It is likely that there is a continuous increase in moisture content with a resultant decrease in modulus in a dorso-palmar direction. This is unlikely to be established *in vivo* as actual zones. The use of zones artificially exaggerates this effect but, on the other hand, the use of zones has shown that this important mechanism is likely to exist.

The results from samples tested at an *in vivo* moisture content must be examined with caution owing to the time taken to prepare samples and also the effect of milling the samples. It is expected that this preparatory work considerably affected the moisture content of the samples, which would then have an influence on the mechanical properties of each zone.

It was, however, interesting to note for samples tested at *in vivo* moisture content that there were no significant differences in moduli between zones 2, 3 and 4 (Figure 7.1). Many authors believe that for horse hoof there is a decreasing gradient of stiffness across the hoof wall and this is likely to be regulated by moisture content (Leach 1980; Bertram and Gosline 1987; Thomason *et al* 1992; Douglas *et al* 1996; Kasapi 1997; Kasapi and Gosline 1997; Kasapi and Gosline 1999). It had previously been shown in Chapter 3 that there was no significant difference in moisture content between zone 3 and 4. Although there was a significant difference in moisture content for zonal beams between zone 2 and zone 3, the 5% difference was not enough to result in a significantly lower modulus.

For horse hoof, Zenker *et al* (1995) thought that the effect of water on the mechanical behaviour of hoof horn provided a mechanism through which the mechanical properties of different areas of the hoof wall can be adjusted to the requirements of the hoof. As water greatly influences the mechanical properties of donkey hoof horn, it is more than likely that this mechanism also exists in the donkey hoof. The lack of a differential in

mechanical properties between zones 2, 3 and 4 for *in vivo* samples may be different to the expected gradient of stiffness, but the higher stiffness of zone 1 still affords protection to the hoof.

There was also no significant difference between the moduli for zones 2, 3 and 4 for fully hydrated samples, this was despite having previously shown a significant difference between hydrated moisture content for all zones (Chapter 4). However, zones 2-4 only showed a change of 8% in moisture content which, again, was not enough to result in a difference in moduli. Zone 1, however, was not capable of absorbing as much moisture as the other zones, resulting in a subsequently higher modulus. Kasapi and Gosline (1997) and Kasapi (1997) also found zonal differences in hydrated moisture contents and attributed this firstly to the contribution of medullary cavities to the amount of hoof horn available for uptake of water. Secondly, there may be possible differences in protein type and content in different areas of the hoof but their work did not examine this area. As was previously found for donkey hoof horn (Chapter 3), the glycosaminoglycans content appears to vary across the HWD and this substance has been shown to have a role in regulating the amount of water in connective tissue (Junquiera 1971). This may therefore explain the differences in the ability of the hoof to take up differing amounts of water across the HWD and in its subsequent effect on modulus. The lower moisture absorption capability of zone 1 would also afford protection from the environment as hooves subjected to excess wetting would not result in absorption of water to the detriment of the stiffness, and consequently the protection and support, afforded by the hoof.

It was initially expected prior to moisture content analyses that fully hydrating the samples would normalise for moisture content by bringing all the zones to a similar level of hydration and therefore resulting in similar mechanical properties across the zones. However, the moisture content analyses and the zonal mechanical testing has shown that this is not possible. This would also apply to samples equilibrated at different relative humidities as moisture uptake for different areas would be different. This fact does not appear to have been considered by workers when equilibrating hoof horn samples at different relative humidities. Küng (1991) carried out tensile testing on

inner and outer wall samples at 65% relative humidity. Although the outer wall had a higher tensile stiffness, it was not significantly higher than the inner wall samples. Zenker *et al* (1995) also equilibrated samples at 65% RH and found differing results for inner and outer wall samples. Moisture content levels were not established by both sets of workers. This underlines the importance of knowing the moisture content of samples when tested as these differences between inner and outer wall samples were likely to be explained by the differing moisture contents.

The results reported here indicated that the best methods for normalising for moisture content to see if there are, indeed, differences in mechanical properties across the HWD were by testing at 38% hydrated regain and by drying samples at room temperature. This resulted in no significant differences between the moduli of any zones for those tested at 38% hydrated regain and for those tested following drying at room temperature. Again, this has not been reported for horse hoof horn. The standard deviations for the samples dried at room temperature were large and this may explain the lack of a difference between zones. Moisture content analyses had previously shown that drying at room temperature was not an ideal method but it was found that drying of samples over phosphorus pentoxide resulted in warping of the samples. This alternative method of drying at room temperature was therefore chosen. Hydrating samples at 38% hydrated regain was therefore the best way of actually testing the mechanical properties of hoof horn *per se* as the level of moisture was believed to be consistent for all zones. It is not known whether or not this also occurs in horse hoof as all the previous studies have not allowed for the influence of moisture content.

Reilly (1999) suggested that the higher cysteine content of IFAPs extracted by Grosenbaugh and Hood (1992a and 1992b) from the outer hoof wall compared to the level in the laminar tissue, may explain the decrease in hoof wall stiffness in a dorso-palmar direction although he did not examine this possible effect. This decrease in stiffness was not, however, shown for donkey hoof horn. Examination of the cysteine content may therefore be useful if micro-mechanical tests could, in fact, identify differences in stiffness between zones.

The effect of a significant change in moisture regain for zones 3 and 4 between *in vivo* samples and 38% hydrated regain samples did not have a significant effect on modulus. This would offer protection to the dermal epidermal junction as a drastic change in moisture content and subsequent mechanical properties of this area may be catastrophic.

Zone 1, however, only showed a difference of an 8% lower moisture content for *in vivo* samples than those samples at 38% hydrated regain but this resulted in a significantly higher modulus for *in vivo* samples. In this way, the outer hoof wall may not be able to cope with continued changes in moisture content resulting from environmental differences which may occur due to present day management systems such as stabling of animals. In effect, the constant changes in moisture content within the environment, cause the moisture gradient within the hoof to change. If this becomes too steep, for example when the outer hoof wall is very dry, this may cause the hoof wall to crack. This mechanism is known to exist for wood (Pratt 1986). Cracks, in turn, threaten the protection of the contents of the hoof.

The moduli results for full HWD samples were similar to those obtained when the moduli for the zonal beams were combined and averaged for both full hydration and dried samples. This was to be expected as the zones contribute to the overall mechanical properties of the hoof wall. It is likely that the much lower modulus for full HWD samples than for *in vivo* zonal samples was due to the moisture loss as these samples took longer to prepare.

It was interesting to note that there was no significant difference for the results shown in Chapter 6 for full hoof wall depth samples between the moduli of samples tested at *in vivo* moisture content and those tested fully hydrated. However, when partial HWD samples were examined, zonal differences in moduli were shown between those tested at *in vivo* moisture content and those tested fully hydrated. These significant differences occurred in zone 1, zone 3 and zone 4. There was no significant difference between moduli for zone 2 at these two levels of moisture content ($p=0.09$, Student's t

test). The analysis of full HWD samples had therefore masked these subtle differences that occur at a zonal level. It would not therefore be appropriate to accept testing of zonal samples at full hydration only.

The results found for zonal fully hydrated moisture contents indicated that there was a difference in water uptake between the different zones. This then resulted in differences in zonal bending results. The moduli results for full hoof wall depth beams at full hydration therefore reflected a compromise between the differences in moisture content between zones and the subsequent differences in mechanical properties across the hoof wall depth. This has not been reported previously for hoof horn.

As mentioned in the literature review at the beginning of the thesis, differences in intermediate filament alignment and volume fraction exist across the HWD of horse hoof horn (Kasapi 1997; Kasapi and Gosline 1999). If these differences do, in fact, exist for donkey hoof, they are likely to be subtle and may not be detected by 3 point bending or probably by any other mechanical means on such large specimens. Tests carried out on micro beams with considerable attention being paid to the moisture content of the samples may detect such differences.

Comparison with previously published 3 point bending results for horse hoof (Hinterhofer 1996; Hinterhofer *et al* 1998) on a zonal basis is difficult due to the type of test carried out, speed of test and methods of hydrating or dehydrating samples, together with methods of calculating moisture contents. Samples from both studies were also pooled from both front and hind hooves and it is not yet known whether this would have an effect on the mechanical properties of hoof horn.

Hinterhofer (1996) and Hinterhofer *et al* (1998) used partial HWD samples. It may be that their samples equated to part of zone 1 together with zone 2 used in this study. However, there was no description of the samples prepared to be tested in the *y* direction. This is confusing as the sample length was 40-50 mm which would be very difficult to achieve with the curved shape of the hoof wall. No mention was made of using curved samples. If this was not the case then sample testing may not have taken

this fact into account and may have covered possibly all zones of the hoof wall. The actual HWH used for removal of the sample was also not indicated. The speed of sample testing by Hinterhofer (1996) was 5 mm min^{-1} whereas that for Hinterhofer *et al* (1998) was 2 mm min^{-1} which were both faster than that used in this study.

The results from two sets of samples used by Hinterhofer (1996) following storage for three weeks and twenty four hours at 2°C were similar to those from zone 1 for samples dried at room temperature and those from an *in vivo* moisture content. The moduli for the remaining samples which were stored for 24 hours at 2°C and tested perpendicular to the tubules were similar to those found for zones 1 and 2 of an *in vivo* moisture content.

The direction of sample testing by Hinterhofer (1996) was not clear and final sample dimensions were slightly different to those used by Hinterhofer (1996). These were 3-4 mm x 4-5 mm x 50 mm. The span to depth ratio was not mentioned but the span was assumed to be the same as in Hinterhofer (1996) of 30 mm. If this was indeed the case then the sample dimensions would be important as a depth of 4 mm would provide a ratio of 7.5 which is less than the ideal of ten. In turn this would increase the underestimate of the moduli by 3%. Hinterhofer *et al* (1998) only tested samples perpendicular to the line of the tubules.

The moduli results from Hinterhofer *et al* (1998) of 762 MPa for samples tested at *in vivo* moisture content are lower than zone 1 but higher than zone 2 in the present study (1223 and 597 MPa respectively). Their samples conditioned at 65% RH are similar to those found for all donkey hoof horn zonal samples dried at room temperature (1525-2083 MPa). There was a considerable range of 1636-8650 MPa for horse hoof samples tested following drying at 110°C. The lower range of these results could be compared with those from samples dried at room temperature.

Other authors that carried out 3 point bending were Wagner *et al* (2001) but they only divided the hoof wall into two. They also only tested samples from longitudinal sections in a dorso-palmar and palmar dorsal direction. It is likely that the outer *SM*

samples from the study of Wagner *et al* (2001) would be the equivalent of zones 1 and 2 and possibly part of zone 3 from this present study. The percentage HWD afforded by the *SMZA* (as defined by Wagner *et al* 2001 in Chapter 1) may be equivalent to part of zone 3 and the whole of zone 4. Again, a direct comparison could not be made between their results and those from the present study for donkey hoof horn owing to the different orientation of samples, together with the lack of information on sample moisture content. The moduli, however, for the outer *SM* found by Wagner *et al* (2001) were similar to zone 2 for fully hydrated samples of donkey hoof horn. Those from the *SMZA* were similar to zone 3 from fully hydrated samples. It may also be that samples had absorbed moisture from their storage in the towelettes soaked in physiologic saline. If the 86.4% relative hydration was the equivalent of 86.4% dry matter, then the moisture content of samples would have been 13.6% which would be very low. This is unlikely to be the case as the moduli would then have been much higher. As mentioned in section 1.11, the span to depth ratios for the samples tested by Wagner *et al* (2001) were low. The strain rate data were not provided but, following calculation of their strain rates, two different strain rates had been used which may have resulted in different moduli for the two areas.

Again, as has been shown above, there are really too many different variables to be able to provide a direct comparison between the mechanical properties of horse and donkey hoof horn. The only way to assess whether there are, indeed differences across the HWD would be to carry out the same tests on horse hoof that have been established in the present study on donkey hoof horn.

This study, together with the literature review, reiterate the importance of the use of clear, correct and standardised methodologies.

What is also clear from these results is that limited information is gained from examining the full HWD, or even if it is divided into two, as so many differences occur across the HWD such as tubule density and moisture content. The hoof is made up of constituent parts and these must be broken down to provide more detail in order to understand how the hoof works as a whole.

7.6 Future Work

A greater number of samples should be used to ensure that these results are, indeed, representative of donkey hoof horn and this experiment should be repeated on horse hoof and other hooved animals.

An increased number of levels of hydration should be used to examine more closely the effect of moisture content on the mechanical properties of donkey hoof horn.

The uptake of moisture by zonal samples following equilibration at different relative humidities should be investigated when using different relative humidities to store samples prior to mechanical testing.

The hoof wall depth could be divided into even smaller zones for analyses of moisture contents and subsequent testing by 3 point bending to see if a difference in mechanical properties can be noted.

Both transverse and longitudinal zonal hoof wall samples should be analysed to take into account the altered span to depth ratios.

A quantitative analysis of glycosaminoglycans across the HWD should be ascertained in order to see whether this corresponds to the ability of the hoof to take up water.

The alignment of intermediate filaments together with the cysteine content across the hoof wall depth should be identified. If these do, indeed, vary then micro-mechanical tests should be carried out on these different areas to assess whether alignment of these filaments or cysteine content do affect the mechanical properties of these areas.

Other methods of sample preparation should be investigated in order to avoid excess moisture loss.

7.7 Conclusions

- Three point bending allowed determination of mechanical properties across the hoof wall depth.

- The use of a consistent level of hydration of 38% hydrated regain or drying samples at room temperature indicated that the mechanical properties did not vary across the four zones tested. This in turn emphasised that the hoof is considerably reliant on moisture content to alter its mechanical properties.

- Fully hydrated samples did not provide a means of normalising for moisture content as uptake of water across the hoof wall depth varied, resulting in differing mechanical properties across the zones.

- A self-protecting mechanism exists whereby the outer part of the hoof wall is unable to absorb as much moisture as the remainder of the wall, leading to increased stiffness of the external surface and therefore affording the hoof protection from the environment.

- Another protective mechanism exists in donkey hoof horn as a high percentage change in moisture content in zones 3 and 4 did not result in a significant change in modulus. However, a low percentage change in moisture content in zone 1 resulted in a significant change in modulus.

- The average of the moduli results for zonal mechanical testing for those samples tested at full hydration and dried samples were similar to the results for full hoof wall depth samples found previously in Chapter 6.

- Comparison of the results from the present study with studies from other workers was difficult owing to type of test, speed of test, methods of hydration and dehydration and calculation of moisture contents.

8. INTERACTIONS

8.1 Introduction

There were important relationships shown in Chapters 6 and 7 between moisture content and mechanical properties of donkey hoof horn. The interactions of tubule density with these parameters and also other variables such as age, bodyweight and hoof wall depth were examined for clippings using correlation and regression analyses. The samples used were those for which tubule density, moisture content and mechanical properties were examined on the same clipping samples. Some individual relationships between two factors, *e.g.* between moisture content and mechanical properties, have been discussed in the individual chapters.

Individual correlations were carried out using the Pearson product moment correlation coefficient as some data were not present for individual animals and a correlation matrix required an equal amount of results for each variable. This occurred when, for example, some samples had warped following drying and could not then be mechanically tested.

8.2 Inter-relationships Between Measured Parameters

The significant positive correlations ($p<0.05$) and regression analyses are shown in Table 8.1. The regression analyses are only mentioned if they were significant ($p<0.05$). It would perhaps be expected that there would be strong correlations between the moduli of samples tested at different levels of hydration as the hoof horn itself is intrinsically the same.

Table 8.1 - Correlations and Regressions for the Results for Clippings for Variables Examined in This Study

Variables	Correlation	p Value	Regression R^2 (%)
HWD v BWT	0.92	p<0.001	85
Hydrated MPa v *In vivo* MPa	0.68	p<0.05	47
Hydrated MPa v Z1 TD	0.77	p<0.05	59
Hydrated MPa v Z2 TD	0.77	p<0.05	59
Hydrated MPa v Z3 TD	0.78	p<0.05	60
75% RH MPa v *In vivo* MPa	0.72	p<0.05	67
75% RH MPa v Hydrated MPa	0.66	p<0.05	60
75% RH MPa v Z1 TD	0.80	p<0.05	65
In vivo MPa v Z1 TD	0.94	p<0.01	88
In vivo MPa v Z2 TD	0.84	p<0.05	70
Hydrated Moisture Content v Z3 TD	-0.80	p<0.05	64
Hydrated Moisture Content v Z4 TD	-0.92	p<0.01	84
TD v Hydrated MPa	0.64	p<0.05	41
TD v 75% RH HMC_D	0.60	p<0.05	36
TD v Z1 TD	0.92	p<0.001	60
TD v Z2 TD	0.93	p<0.001	91
TD v Z3 TD	0.74	p<0.001	65
TD v Z4 TD	0.76	p<0.01	58
Z1 TD v Z 2 TD	0.86	p<0.05	73
Z2 TD v Age	0.61	p<0.05	NS
Z2 TD v Z1 TD	0.91	p<0.001	70
Z2 TD v Z3 TD	0.68	p<0.05	NS
Z2 TD v Z4 TD	0.63	p<0.05	NS
Z3 TD v Z4 TD	0.88	p<0.001	81

Key:

HWD	Hoof wall depth
BWT	Bodyweight
TD	Tubule density (full hoof wall depth)
Hydrated MPa	Moduli at full hydration
75% RH MPa	Moduli following equilibration at 75% RH
Z1/2/3/4TD	Zones 1-4 Tubule density
HMC_D	Hydrated regain
NS	Not significant

8.3 Bodyweight

As has already been mentioned in Chapter 2, there was a very strong positive correlation between hoof wall depth and animal bodyweight of 0.92 ($p<0.001$). A regression analysis resulted in an R^2 value of 85%. This implies that a feedback mechanism may exist which alters the HWD according to bodyweight. This may be a stress factor imposed on the dermal papillae which, in turn, respond to the weight of the animal. It would be interesting to see whether this relationship does occur for horses. If the dermal papillae do respond to bodyweight then this shows the importance of maintaining a full HWD. Unfortunately the HWD is often reduced by excessive rasping of the wall (Hopegood, L. personal observations), thus reducing the hoof's capability of protecting the inner sensitive structures and supporting the whole animal. Reilly (1999) found a significant negative correlation between bodyweight and zonal tubule density and between bodyweight and *in vivo* stiffness but this did not exist for donkey hoof horn. The lack of differences for the present study may be because the zonal boundaries were slightly different to those used by Reilly (1999) or that there was, indeed, a species difference.

8.4 Tubule Density

8.4.1 Tubule Density - Full Hoof Wall Depth

For this present study there was no relationship found between moisture content and tubule density for full HWD samples. There was, however, a modest positive correlation between tubule density for the full HWD and the moduli of samples tested at full hydration of 0.64 ($p<0.05$). A regression analysis resulted in an R^2 value of 41%. This would suggest that tubule density does affect the amount of water that hoof horn can absorb and, in turn, this affects the modulus. As tubule density is ascertained by counting the number of medullae per cross sectional area, this relationship may be linked to the number of medullae that are present that may provide free space for attachment of water. A similar reason may exist for there

being a modest positive correlation between tubule density for the full HWD and the hydrated regain of samples equilibrated at 75% RH of 0.60 ($p<0.05$).

However, if this is indeed the case, other relationships of tubule density to moisture contents would have been expected. It must be borne in mind, however, that the tubule density value is for the full HWD and there has been shown to be considerable changes across the HWD in tubule density. It may be that tubules play a greater role when there is a limited amount of water present as, for example, when samples were equilibrated at 75% RH. This does not, however, hold true with the relationship between tubule density and the hydrated moduli. It would have been expected that there would also have been a relationship between tubule density and hydrated moisture content for the full HWD but this was not shown. A zonal relationship will, however, be discussed in the next section.

The relationships between tubule density for the full HWD and those for individual zones may be expected as the full HWD relies on the contribution from individual zones.

8.4.2 Zonal Tubule Density

There was a number of relationships found between the zonal tubule density results and the mechanical properties of donkey hoof horn. These were predominantly shown for fully hydrated tests. It should be borne in mind, however, that the moduli are for full HWD samples.

There were strong correlations between the moduli for hydrated samples and tubule density for zones 1, 2 and 3, with regression analyses resulting in R^2 values of 59%, 59% and 60% respectively.

There were very strong correlations between the moduli for *in vivo* samples and tubule density for zones 1 and 2, with regression analyses resulting in R^2 values of 88% and 70% respectively.

The other link between zonal tubule density and stiffness was found for zone 1 and samples tested following equilibration at 75% RH with a positive correlation of 0.80 ($p<0.05$) and a regression analysis R^2 value of 63%. These results imply that an increase in the tubule density results in an increase in hoof stiffness. This may act as a protective mechanism, particularly when hoof horn is sustained in water for long periods of time, such as in adverse weather conditions. The resultant increase in stiffness may decrease the chances of collapse of the hoof horn following excess wetting.

There were strong negative correlations between the hydrated moisture content and the tubule density for zones 3 and 4, with R^2 values of 64% and 84% respectively. This correlation implies that an increase in tubule density results in a decreased hydrated moisture content. This is, however, contrary to the belief that the relationship between tubule density and moisture content may be linked to the number of medullae that provide free space for attachment of water. Reasons for this relationship are unclear at the present time. Further investigation would need to examine the moisture content of partial HWD samples with tubule density and mechanical properties.

There were strong relationships between mean tubule density and zonal tubule density and also between the tubule density of different zones. This may be expected as the zonal tubule density results obviously contribute to the final overall tubule density.

The tubule density for zone 2 appeared to have particular relationships with all the remaining zones whereas the number of relationships does not exist for other zones. This area is still an area of decreasing tubule density in a dorso-palmar direction which differs to that of zone 3 and zone 4 which showed a relatively consistent tubule density. It was interesting to note points related to zone 2 for pony hoof horn. The correlation was highest in zone 2 between bodyweight and tubule density (Reilly 1999). The size of the tubule cortex in this area was also implicated in aiding

the weight bearing mechanism (Reilly 1999). It may be therefore that this area holds particular significance for both donkey and horse hoof horn. The reason for the occurrence of these relationships is, however, unclear.

8.5 Mechanical Properties

The main relationships of the mechanical properties of donkey hoof horn with moisture content and tubule density have been mentioned above in section 8.4.

The moduli at the following levels of hydration were related: fully hydrated, *in vivo* and samples subjected to 75% RH. This was to be expected as there is such a close relationship between moisture content and mechanical properties of donkey hoof horn.

8.6 Further Comparisons

Table 8.2 outlines the multiple regression equations for results from clipping samples in this study.

Table 8.2 - Multiple Regression Equations for the Results for Clippings for Factors Examined in This Study

Multiple Regression Equation	R^2 (%)	p Value
HWD = 2.91 + 0.0327 BWT - 0.271 Age	95	p<0.01
HWD = 2.16 + 0.0302 BWT - 0.00565 Hydrated MPa	88	p<0.05
HWD = 1.78 - 0.0145 Hydrated regain + 0.0316 BWT	85	p<0.05
Hydrated MPa = -186 + 25.7 TD + 0.0359 Dry MPa	87	p<0.05
75% Hydrated regain = -3.55 + 0.00909 BWT + 0.238 Hydrated regain	87	p<0.05
75% Hydrated regain = -3.75 + 0.244 HWD + 0.243 Hydrated regain	84	p<0.05
Hydrated regain = 4.3 + 1.94 75% Hydrated regain + 0.669 Moisture regain	86	p<0.05
Hydrated regain = 21 + 3.5 75% Hydrated regain - 0.0309 BWT	83	p<0.05
TD = 6.72 - 0.142 Age + 0.0291 Hydrated MPa	79	p<0.05

For key, see Table 8.1.

Again, the multiple regressions indicate a link of bodyweight with HWD. With the added influence of age, the R^2 value has increased from 85% to 95%. Although there was no direct influence, this implies that there may be an influence of age on HWD.

9. ENUMERATED CONCLUSIONS

The work described in this thesis has made original contributions to scientific endeavour and to knowledge in the following ways:

1. The study is unique in that it has applied quantitative techniques to the analysis of donkey hoof horn.

2. Difficulties in comparing results with those from the literature have been identified. This is because many different techniques have been used for the collection, storage and preparation of hoof horn for analysis.

3. The study has established protocols for the collection, storage and preparation of donkey hoof horn for analysis.

4. A standardised protocol of drying samples over phosphorus pentoxide to assess the moisture content of donkey hoof horn was selected following assessment of nine different methods of dehydrating hoof horn.

5. The study uniquely identified the moisture content of donkey hoof horn as 33% and found that it was significantly higher than the 26% shown for horse hoof horn. This indicated a difference between the two species.

6. Moisture content analysis has not previously been carried out across four zones of the *Stratum medium* of hoof horn. From these results it was shown that moisture content is seen to increase across the *Stratum medium* in a dorso-palmar direction for both donkey and horse hoof horn.

7. The work in this thesis was unique in its field in that it assessed the moisture content of donkey hoof horn at four different levels of hydration: *in vivo*, fully hydrated, dried and following storage at 75% relative humidity.

8. The moisture content of donkey hoof horn is very important as the hoof wall functions at 91% of the full saturation level of hoof horn.

9. Determination of the sorption isotherm for donkey hoof horn had not previously been carried out using a large number of relative humidity environments. Determination of the desorption isotherm had not previously been carried out.

10. The sorption and desorption isotherms for donkey hoof horn showed similar results to other keratinised tissues such as wool and horn.

11. It was the first time that relative humidity environments had been used to attempt to rehydrate samples of donkey hoof horn to an *in vivo* moisture content. This was not, however, achieved at even very high levels of humidity. This must therefore be borne in mind when mechanically testing samples following equilibration at different levels of humidity as these would always be at a lower moisture content than an *in vivo* level.

12. A three-zoned pattern of tubule density is suggested for donkey hoof horn which contrasts with the four-zoned pattern of tubule density which has been reported for both pony and horse hoof horn and was thought to represent an equid pattern.

13. Slight zonal differences were seen between tubule density for clipping and morbid samples of donkey hoof horn. Clippings should therefore be used with caution but have provided a very useful tool to establish protocols and provide preliminary data for donkey hoof horn.

14. A relationship between hoof wall depth and bodyweight of donkey has not been reported previously.

15. The work in this thesis was unique in its field in that it assessed the mechanical properties of donkey hoof horn using a 3 point bending technique at four different levels of hydration: *in vivo*, fully hydrated, dried and following storage at 75% relative humidity. There was an inverse relationship between moisture content and modulus.

16. The lack of a significant difference between the moduli of samples tested at *in vivo* moisture content and those tested fully hydrated had not been reported previously and many indicate a "fail safe" mechanism whereby there is a level of hydration beyond which there is no further change in hoof stiffness.

17. Four levels of hydration have also not been used before to assess the modulus of zonal beams of hoof horn.

18. This is the first time that the mechanical properties of hoof horn have been shown not to vary across the four zones when the same level of hydration was used for each of the zones.

19. A self-protection mechanism was identified whereby the outer part of the hoof wall is unable to absorb as much moisture as the remainder of the hoof wall, leading to an increased stiffness in the external surface and thus providing the hoof with protection from the environment.

20. Tubule density influenced the hydrated regain of samples following equilibrium at 75% RH. Zonal tubule density also influenced the hydrated moisture content of donkey hoof horn.

21. Inter-relationships were shown between tubule density and the mechanical properties of donkey hoof horn for both zonal and full hoof wall depth samples.

22. What is clear from these results is that limited information is gained from examining the full HWD, or even if it is divided into two, as so many differences

occur across the HWD such as tubule density and moisture content. The hoof is made up of constituent parts and these must be broken down to provide more detail in order to understand how the hoof works as a whole.

23. It is suggested that the future examination of the *Stratum medium* of donkey hoof horn for tubule density or moisture content should be conducted using three zones, rather than the four zones previously found for pony and horse hoof horn.

10. SUMMARY OF FUTURE RESEARCH DIRECTIONS

Following the results presented in this thesis together with the implications of these results which were discussed in individual chapters, a summary of suggested future research directions is now given:

1. Repeat studies on morbid capsules for a known donkey population, both from the UK and from abroad in order to assess whether the results found in the work for this thesis provide similar results for morbid samples and for those from donkeys from their indigenous environment.

2. Repeat studies for horses and other hooved animals are recommended in order to assess whether the responses found in this study are similar across the hooved species.

3. A "map" of moisture content, tubule density and mechanical properties around the whole hoof capsule for both donkeys and other hooved animals would enable the effects of nutrients or different management regimes on these characteristics to be assessed.

4. Quantitative analyses of "normal" hoof horn has provided invaluable data for comparative studies with, for example, animals with known foot problems or with diseased hoof horn.

5. The use of more sophisticated approaches in the study of the moisture content of donkey hoof horn may provide an insight into where water molecules are sited within samples. This may reveal reasons as to how water acts as a plasticiser in hoof horn. Advanced techniques to include could be the use of differential scanning calorimetry, electron paramagnetic resonance spectroscopy, nuclear magnetic resonance spectroscopy and infra-red spectroscopy.

6. Various questions still need to be addressed. For example, as to whether, for donkey hoof horn, tubule morphometry, intermediate filaments, protein and glycosaminoglycans content are, in part, responsible for the differences in moisture content of hoof horn across the *Stratum medium*.

7. An "optimal" level of donkey hoof horn hydration is yet to be discovered. Continuation of research into this field may provide the answers to ensure that management procedures enable the functions of the hoof to be achieved.

8. One way in which this method of ascertaining tubule density could be improved is to use an overlay grid that follows the curve of the inner hoof wall. Wear or damage to the toe area would then not influence the curve of the grid. The tubule population at the edge of the hoof wall, together with that near the *Stratum internum* that is missed by the present grid, would then be taken into account. This would also allow the remaining counts to follow the shape of the hoof wall. From a practical point of view, however, this may be difficult to achieve as the curve of the wall varies between animals. Individual grids would therefore need to be established.

9. A more detailed count may be achieved by enlarging the image to a size that would enable an overlay grid to be used that would allocate 1% HWD to each cell. In practice, however, this may prove difficult as, for example, an 8 mm HWD would have to be divided into 100 counting cells.

10. The results from the present study on donkey hoof horn can now be input into the computer model of donkey hoof (Newlyn *et al* 1998) as moduli results were previously used from horse hoof.

11. Donkey hoof horn should be tested both in compression and tension and through different planes to see if it is, indeed, anisotropic.

12. The uptake of moisture by zonal samples following equilibration at different relative humidities should be investigated when using different relative humidities to store samples prior to mechanical testing.

13. The hoof wall depth could be divided into even smaller zones for analyses of moisture contents and subsequent testing by 3 point bending to see if a difference in mechanical properties can be noted.

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Appendix 1 - Donkey Samples Used for Specific Experiments (continued on next page)

Donkey	Moisture Loss Over Time Section 2.3.3	Comparison of Drying Methods Section 2.3.4	Zonal Moisture Contents Section 3.3.1	Hydrated Moisture Content - Full HWD Section 4.3.1	Zonal Hydrated Moisture Contents Section 3.3.1
1	✓	✓	✓		✓
2	✓	✓		✓	
3		✓	✓		✓
4		✓			
5		✓			
6		✓	✓	✓	✓
7		✓			
8	✓	✓			
9	✓	✓			
10		✓	✓		✓
11		✓			
12		✓			
13		✓			
14		✓			
15		✓			
16		✓	✓		✓
17		✓	✓		✓
18		✓			
19		✓			
20		✓			
21		✓		✓	
22		✓			
23		✓			
24	✓	✓	✓		✓
25		✓			
26		✓	✓	✓	✓
27		✓			
28		✓			
29		✓	✓		✓
30		✓		✓	
31		✓	✓		✓

Appendix 1 (continued)

Donkey	Relative Humidity Environments Section 4.3.3	Tubule Density Clippings Section 5.3.1	Bending - Full Hoof Wall Depth Section 6.3.4	Zonal Mechanical Testing Section 7.3.1.4
1	✓	✓	✓	
2		✓	✓	
3				✓
4	✓	✓	✓	
5		✓	✓	
6		✓	✓	
7	✓			✓
8	✓	✓	✓	
9		✓	✓	
10	✓			✓
11	✓			✓
12	✓			
13	✓			
14				
15				✓
16				
17				
18				
19		✓	✓	
20				
21		✓	✓	
22		✓	✓	
23				
24	✓	✓	✓	
25		✓	✓	
26		✓	✓	
27		✓	✓	
28				
29	✓	✓	✓	
30				
31				

Appendix 2 - Moisture Content Results for Different Drying Techniques

Donkey	P_2O_5 (%)	Freeze Drying (%)	Room Temperature (%)	Vacuum Drying (%)	120°C (%)	110°C (%)	105°C (%)	100°C (%)	90°C (%)
1	31.15	31.59	27.01	33.09	35.55	33.71	31.90	10.24	11.59
2	35.72	37.40	26.98	10.36	35.82	34.90	36.03	36.84	32.92
3	32.43	33.13	27.77	6.69	36.92	35.03	10.59	19.86	25.51
4	30.91	29.19	24.69	19.65	33.53	31.10	17.46	31.07	34.29
5	32.30	32.97	28.24	6.30	35.43	27.47	32.84	33.67	29.76
6	35.28	35.01	29.36	30.91	36.15	36.57	38.50	32.31	14.29
7	34.83	34.89	27.52	36.31	36.38	38.31	21.37	32.28	14.25
8	31.93	32.37	27.39	12.36	34.72	33.44	30.96	33.55	25.62
9	32.03	33.48	27.08	35.69	37.01	33.86	35.01	34.94	25.82
10	31.28	28.41	24.41	25.40	33.28	33.89	34.64	26.98	33.00
11	35.58	37.71	31.45	27.41	37.87	35.65	32.70	34.24	25.48
12	35.51	37.38	28.38	17.07	39.06	37.45	36.69	31.62	38.12
13	33.65	34.93	27.25	10.25	34.77	34.40	34.12	30.66	27.71
14	31.13	31.01	26.97	13.27	34.67	33.80	32.15	28.73	26.55
15	34.57	37.24	28.56	31.51	37.44	37.19	28.96	34.23	35.49
16	32.89	35.07	28.93	29.87	35.74	35.76	35.44	34.75	28.94
17	31.49	32.58	22.60	10.48	34.64	30.19	33.75	12.59	34.54
18	37.11	35.65	14.50	23.95	39.12	37.45	37.29	33.43	31.77
19	31.94	31.38	25.89	32.20	35.34	33.80	35.46	31.91	32.09
20	30.37	30.97	23.05	35.50	36.94	34.06	36.58	31.71	34.96
21	36.46	33.20	29.74	17.36	36.30	37.08	36.89	31.17	33.50
22	32.95	33.81	23.09	34.06	36.59	34.53	35.95	33.98	10.26
23	34.29	33.54	27.94	31.61	35.62	34.95	33.03	33.94	35.49
24	32.25	32.09	26.14	27.16	38.97	33.79	32.29	34.67	25.58
25	35.35	35.12	30.06	32.67	38.69	36.15	38.09	27.45	27.92
26	31.35	30.78	26.63	17.35	36.14	33.19	30.73	32.62	23.31
27	32.97	30.45	21.93	23.36	34.07	32.20	34.23	14.30	30.42
28	35.45	31.90	30.58	28.44	36.55	35.08	35.33	34.16	19.11
29	28.83	26.25	17.87	7.50	32.97	29.85	33.54	27.10	13.74
30	34.88	34.20	27.72	10.47	35.55	36.46	34.84	32.72	36.18
31	31.96	33.91	26.25	24.53	35.76	28.71	33.83	11.01	24.44
Mean	33.19	33.15	26.32	22.67	36.05	34.19	32.62	29.31	27.18
SD	2.05	2.69	3.57	9.94	1.62	2.61	5.97	7.50	7.79
CV	0.06	0.08	0.14	0.44	0.05	0.08	0.18	0.26	0.29
Median	32.89	33.20	27.08	24.53	35.82	34.40	34.12	32.28	27.92
Range	29-37	26-38	14-31	6-36	32-39	27-38	11-39	10-37	10-38
P value	0.086	0.837	0.002	0.000	0.514	0.058	0.000	0.000	0.000
Normal?	Y	Y	N	N	Y	Y	N	N	N

Appendix 3 - Full Data Set for the Effect of Age, Gender and Hoof Pigment on the Moisture Content of Donkey Hoof Horn

	Animal Age	Moisture Content (%)	Moisture Content Females (%)	Moisture Content Males (%)	Moisture Content *Non-* pigmented (%)	Moisture Content Pigmented (%)
	9	36	36	37	32	
	8	37	31	32	35	
	8	32	35	32	32	
	5	31	32	32	35	
	5	35	32	36	35	
	5	32	35	35	32	
	5	32	36	35	34	
	5	32	30	33		36
	5	36	32	36		37
	5	35	29	33		31
	5	32	35	32		32
	5	35		33		32
	5	35		31		36
	5	33		35		32
	5	36		31		35
	6	33		34		33
	6	32		34		36
	4	33		31		33
	6	31		31		33
	7	35		31		31
	6	36				35
	7	31				36
	5	34				31
	5	34				34
	7	30				30
	4	31				31
	5	31				31
	5	32				32
	3	31				31
	3	29				29
	3	35				35
Mean	5	33	33	33	34	33
SD	1.41	2.05	2.39	1.90	1.47	2.20

Appendix 4 - Data Set for Moisture Content and Moisture Regain for both Donkey and Horse Hoof Horn

	Moisture Content Donkey (%)	Moisture Regain Donkey (%)	Moisture Content Horse (%)	Moisture Regain Horse (%)
	31	45	28	39
	36	56	31	44
	32	48	28	39
	31	45	30	43
	32	48	21	27
	35	55	22	29
	35	53	22	28
	32	47	22	28
	32	47	27	37
	31	46	32	47
	36	55	26	35
	36	55	27	38
	34	51	27	37
	31	45	24	32
	35	53	29	40
	33	49	27	38
	31	46		
	37	59		
	32	47		
	30	44		
	36	57		
	33	49		
	34	52		
	32	48		
	35	55		
	31	46		
	33	49		
	35	55		
	29	41		
	35	54		
	32	47		
Mean	33	50	26	36
SD	2.05	4.60	3.33	6.10

Appendix 5 - Median Zonal Moisture Contents for Donkey Hoof Horn - 90% Hoof Wall Depth Samples

	Zone 1	Zone 2	Zone 3	Zone 4
	19	26	37	38
	21	29	37	38
	23	33	34	35
	23	33	38	41
	24	34	40	40
	25	36	39	40
	26	37	39	39
	21	29	39	39
	26	34	38	40
	21	29	36	37
Median	23	33	38	39

Appendix 6 - Median Zonal Moisture Contents for Horse Hoof Horn

	Zone 1	Zone 2	Zone 3	Zone 4
	20	23	25	35
	25	27	31	36
	24	27	35	37
	39	32	26	23
	17	20	23	28
	16	21	24	28
	18	19	23	26
	17	18	20	22
	23	25	26	34
	25	27	30	35
	22	25	28	34
	17	23	29	32
	21	24	28	33
	22	25	30	34
	19	21	25	26
	26	28	30	36
	23	24	28	35
Median	22	24	28	34

Appendix 7 - Horse Hoof Horn Full Hoof Wall Depth Samples - Hydrated Moisture Content and Hydrated Regain

	Hydrated Moisture Content (%)	Hydrated Regain (%)
	30	42
	31	46
	30	44
	31	45
	28	40
	29	40
	28	39
	27	37
	29	41
	33	50
	29	41
	30	43
	29	41
	29	42
	31	45
	29	40
Mean	30	42
SD	1	3
CV	5	7

Appendix 8 - Zonal Hydrated Moisture Content and Hydrated Regain for Donkey Hoof Horn

	Hydrated Moisture Content (%)				Hydrated Regain (%)			
	Zone 1	**Zone 2**	**Zone 3**	**Zone 4**	**Zone 1**	**Zone 2**	**Zone 3**	**Zone 4**
	25	30	42	43	33	44	71	76
	27	35	42	43	36	53	72	75
	30	40	42	43	43	67	71	75
	23	36	40	44	30	56	68	77
	28	38	43	44	40	60	77	78
	30	39	41	43	43	64	71	75
	28	40	40	45	40	66	68	81
	24	31	40	41	32	45	67	70
	29	37	40	42	41	58	66	72
	26	32	39	41	35	47	63	69
Mean	27	36	41	43	37	56	69	75
Median	28	36	41	43	38	57	69	75
SD	2	4	1	1	5	9	4	4
CV (%)	9	10	3	3	12	16	5	5

Appendix 9 - Horse Hoof Horn - Zonal Hydrated Regain

	Zone 1	Zone 2	Zone 3	Zone 4
	38	37	39	66
	41	43	49	64
	53	53	78	93
	69	51	39	40
	36	33	39	59
	32	35	42	56
	39	35	40	54
	35	33	40	57
	37	37	39	59
	43	47	46	59
	37	39	45	72
	30	35	49	67
	39	38	46	60
	36	37	48	62
	36	38	46	61
	43	45	47	70
	35	36	42	62
Mean	40	40	46	62
Median	37	37	45	61
SD	9	6	9	11
CV (%)	23	15	20	17

Appendix 10 - Donkey Clipping Samples - Data Set for Tubule Density and Percentage Hoof Wall Depth (continued on next page)

Donkey															
1		2		8		10		13		14		17		19	
%HWD	TD	%HWD	TD	%HWD	TD	%HWD	TD	%HWD	TD	%HWD	TD	%HWD	TD	%HWD	TD
5	34	6	16	3	31	3	22	5	23	5	24	4	11	5	26
10	35	12	13	7	22	7	23	11	19	9	22	9	9	10	18
14	34	18	10	10	17	10	24	16	16	14	20	13	9	15	13
19	26	24	9	13	13	13	18	21	14	18	18	17	8	20	13
24	21	29	8	17	14	17	15	26	12	23	15	22	8	25	10
29	12	35	8	20	12	20	16	32	8	27	11	26	8	30	8
33	11	41	9	23	10	23	13	37	8	32	9	30	7	35	9
38	12	47	7	27	11	27	10	42	11	36	11	35	8	40	9
43	10	53	9	30	10	30	11	47	10	41	9	39	8	45	10
48	9	59	9	33	8	33	11	53	8	45	9	43	7	50	11
52	10	65	7	37	8	37	11	58	9	50	8	48	7	55	10
57	13	71	9	40	6	40	9	63	8	55	8	52	6	60	9
62	10	76	8	43	6	43	7	68	10	59	7	57	7	65	10
67	9	82	9	47	7	47	8	74	10	64	8	61	7	70	10
71	11	88	9	50	8	50	7	79	10	68	8	65	6	75	10
76	10	94	8	53	8	53	7	84	8	73	8	70	8	80	9
81	9	100	8	57	8	57	7	89	10	77	6	74	7	85	9
86	9			60	7	60	8	95	8	82	8	78	7	90	10
90	10			63	8	63	9	100	10	86	7	83	7	95	10
95	11			67	7	67	9			91	8	87	7	100	10
100	12			70	7	70	10			95	9	91	8		
				73	8	73	9			100	9	96	7		
				77	9	77	10					100	8		
				80	8	80	9								
				83	9	83	9								
				87	8	87	11								
				90	8	90	10								
				93	9	93	10								
				97	9	97	11								
				100	9	100	10								

Appendix 10 (continued)

Donkey													
20		22		23		24		26		30		31	
%HWD	TD	%HWD	TD	%HWD	TD	%HWD	TD	%HWD	TD	%HWD	TD	%HWD	TD
6	17	3	16	4	20	5	10	4	12	3	33	4	16
11	14	6	12	7	19	10	11	9	13	6	25	9	14
17	12	8	10	11	14	14	11	13	12	10	23	13	11
22	11	11	11	15	15	19	11	17	9	13	17	17	10
28	8	14	11	19	16	24	9	22	7	16	15	22	13
33	10	17	10	22	15	29	9	26	8	19	13	26	8
39	9	19	10	26	12	33	8	30	8	23	12	30	9
44	10	22	10	30	13	38	7	35	8	26	10	35	9
50	9	25	10	33	10	43	8	39	8	29	12	39	8
56	10	28	8	37	9	48	7	43	8	32	10	43	9
61	9	31	8	41	11	52	8	48	8	35	11	48	7
67	9	33	8	44	9	57	7	52	8	39	8	52	8
72	10	36	6	48	10	62	10	57	8	42	9	57	10
78	10	39	6	52	10	67	8	61	8	45	8	61	9
83	10	42	6	56	9	71	9	65	7	48	7	65	10
89	10	44	7	59	8	76	9	70	7	52	8	70	10
94	9	47	7	63	11	81	8	74	7	55	8	74	9
100	10	50	6	67	10	86	8	78	7	58	8	78	8
		53	6	70	10	90	7	83	7	61	7	83	9
		56	7	74	10	95	8	87	7	65	7	87	8
		58	5	78	10	100	8	91	8	68	8	91	8
		61	6	81	10			96	8	71	7	96	9
		64	7	85	10			100	8	74	9	100	10
		67	5	89	9					77	8		
		69	6	93	12					81	9		
		72	5	96	13					84	9		
		75	7	100	12					87	8		
		78	7							90	9		
		81	5							94	10		
		83	7							97	8		
		86	7							100	10		
		89	6										
		92	7										
		94	8										
		97	9										
		100	8										

Appendix 11 - Donkey Morbid Samples - Data Set for Tubule Density and Percentage Hoof Wall Depth

Donkey																	
1		2		3		4		5		6		7		8		9	
%HWD	TD	%HWD	TD	%HWD	TD	%HWD	TD	%HWD	TD	%HWD	TD	%HWD	TD	%HWD	TD	%HWD	TD
4	34	4	26	4	27	6	27	4	28	4	25	5	34	5	28	4	30
9	34	7	24	8	23	12	24	9	23	8	25	10	30	11	21	8	26
13	29	11	21	12	18	18	21	13	19	12	24	15	24	16	18	12	20
17	27	14	18	17	16	24	17	17	14	16	19	20	21	21	16	15	17
22	26	18	16	21	16	29	15	22	13	20	20	25	16	26	16	19	15
26	20	21	15	25	13	35	13	26	13	24	15	30	14	32	15	23	14
30	15	25	12	29	14	41	13	30	11	28	12	35	14	37	12	27	11
35	12	29	11	33	12	47	12	35	10	32	9	40	13	42	11	31	12
39	10	32	11	37	9	53	10	39	9	36	10	45	10	47	11	35	10
43	8	36	10	42	10	59	12	43	9	40	8	50	10	53	10	38	10
48	8	39	10	46	9	65	12	48	8	44	8	55	9	58	9	42	10
52	10	43	7	50	8	71	13	52	8	48	9	60	9	63	8	46	8
57	10	46	8	54	7	76	13	57	8	52	8	65	10	68	9	50	9
61	10	50	8	58	9	82	11	61	8	56	6	70	10	74	9	54	7
65	9	54	9	62	8	88	11	65	8	60	8	75	9	79	8	58	7
70	9	57	8	67	9	94	10	70	8	64	9	80	10	84	8	62	8
74	10	61	7	71	8	100	11	74	7	68	8	85	11	89	9	65	8
78	10	64	7	75	9			78	9	72	8	90	11	95	9	69	8
83	10	68	7	79	8			83	9	76	8	95	11	100	10	73	8
87	10	71	7	83	8			87	9	80	7	100	11			77	8
91	9	75	7	87	8			91	8	84	7					81	9
96	10	79	7	92	9			96	8	88	8					85	9
100	11	82	7	96	9			100	8	92	7					88	8
		86	7	100	10					96	8					92	8
		89	7							100	8					96	9
		93	7													100	9
		96	8														
		100	7														

Appendix 12 - Mean Moduli of Elasticity for Bend 2 at Different Levels of Hydration for Zonal Bending

	In vivo Moisture Content (MPa)				Full Hydration (MPa)				38% Hydrated Regain (MPa)				Room Temperature (MPa)			
	Zone				Zone				Zone				Zone			
Donkey	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
3	2122	1172	515	729	642	311	136	160	642	765	516	339	3600	1993	2210	1759
7	812	339	259	555	243	100	79	191	243	189	127	295	1435	882	933	1749
10	996	1024	411	510	279	86	113	143	279	252	614	623	2235	2088	1821	2353
11	602	91	436	195	162	60	70	91	162	119	244	226	1390	1411	1368	782
15	1581	360	367	334	505	285	66	40	505	661	218	246	1754	2371	1547	983
Mean	1223	597	397	465	366	168	93	125	366	397	344	346	2083	1749	1576	1525
SD	621	472	95	206	200	119	30	60	200	294	209	161	913	597	479	640
CV %	51	79	24	44	55	71	33	48	55	74	61	47	44	34	30	42

Appendix 13 - Publications and Conference Proceedings

Some of the following are included for information at Appendix 14.

REILLY, J.D. (1997) My work was presented at the BEVA/Farriers' association meeting. Stoneleigh.

COLLINS, S.N., COPE, B.C., HOPEGOOD, L., LATHAM, R.J., LINFORD, R.G., REILLY, J.D. (1998) Stiffness as a function of moisture content in natural materials: Characterisation of hoof horn samples. *Journal of Materials Science* **33**, 5185-5191.

NEWLYN, H.A., COLLINS, S.N., COPE, B.C., HOPEGOOD, L., LATHAM, R.J. AND REILLY, J.D. (1998) Finite Element Analysis of static loading in donkey hoof wall. *Equine vet. J. Suppl.* **26**, 103-110

REILLY, J.D., COLLINS, S.N., COPE, B.C., HOPEGOOD, L. AND LATHAM, R.J. (1998) Tubule density of the *Stratum medium* of horse hoof. *Equine vet. J., Suppl.* **26**, 4-9.

REILLY, J.D., HOPEGOOD, L., GOULD, L. AND DEVISMES, L. (1998) Effect of a supplementary dietary evening primrose oil mixture on hoof growth, hoof growth rate and hoof lipid fractions in horses: a controlled and blinded trial. *Equine vet. J., Suppl.* **26**, 58-65.

COPE, B.C., HOPEGOOD, L., LATHAM, R.J., LINFORD, R.G., REILLY, J.D., SYMONS, M.C.R., TAIWO, F.A. (1997) Studies of equid hoof horn material by electron paramagnetic resonance. *Journal of Materials Chemistry.* **8**, 1, 43-45.

COPE, B.C., HOPEGOOD, L., LATHAM, R.J., LINFORD, R.G., REILLY, J.D. (1998) Equid hoof horn: a natural engineering composite. In: *Materials' Congress 1998. Some Critical Issues in Biomedical Materials: Materials Solutions to Nature's Design.* 8 April 1998, Royal Agricultural College, Cirencester, Institute of Materials, Abstract 1.5. and presentation.

REILLY, J.D., COLLINS, S.N., COPE, B.C., HOPEGOOD, L. AND LATHAM, R.J. (1998) Properties of the hoof wall and response to nutrition. Presented at "From Mouth to Foot" - The International Research Conference on Equine Laminitis, 9 September 1998.

REILLY, J.D., COLLINS, S.N., COPE, B.C., HOPEGOOD, L. AND LATHAM, R.J. (1998) Laminitis and the hoof horn capsule. Presented at "From Mouth to Foot" - The International Research Conference on Equine Laminitis, 10 September 1998.

"No Hoof, No Donkey" - part of Donkey Sanctuary poster at BEVA 1998

TRAWFORD, A.F. (1998) Multi-disciplinary collaboration for the world-wide benefit of working equines. *In: 3er Coloquio Internactional sobre Equidos de Trabajo.* Mexico, 271-280.

NEWLYN, H.A., COLLINS, S.N., COPE, B.C., HOPEGOOD, L., LATHAM, R.J. AND REILLY, J.D. (1999) Equid Hoof Horn: A Natural Composite. Poster Presentation. 5th

IOM International Conference - Deformation and Fracture of Composites. 18-19 March 1999.

NEWLYN, H.A., COLLINS, S.N., COPE, B.C., HOPEGOOD, L., LATHAM, R.J. AND REILLY, J.D. (1999) Equid Hoof Horn: A Natural Composite. *Proceedings of the 5th International Conference on Deformation and Fracture of Composites, 18-19 March 1999*, IOM Communications Ltd, 231-240.

COLLINS, S.N., WEALLEANS, H., HOPEGOOD, L., LATHAM, R.J., NEWLYN, H.A. AND REILLY, J.D. (2002) Current studies on the donkey hoof. *CPD, Medicine and Surgery of the Donkey, BEVA*, University of Glasgow.

REILLY, J.D., NEWLYN, H., COPE, B., LATHAM, R.J., COLLINS, S. AND HOPEGOOD, L. (2002a) A comparison of different moisture-loss methods for measuring hoof wall moisture content. *Proceedings of the 12th International Symposium on Lameness in Ruminants*, Orlando, Florida, Jan 9-13, 193.

REILLY, J.D., NEWLYN, H., COPE, B., LATHAM, R.J., COLLINS, S. AND HOPEGOOD, L. (2002b) Comparison of growth and growth rates in biotin and evening primrose oil supplemented hooves, from controlled and blinded trials. *Proceedings of the 12th International Symposium on Lameness in Ruminants*, Orlando, Florida, Jan 9-13, 273.

REILLY, J.D., NEWLYN, H., COPE, B., LATHAM, R.J., COLLINS, S. AND HOPEGOOD, L. (2002c) Lipid chemistry of the normal equine hoof and responses to dietary supplementation with evening primrose oil. *Proceedings of the 12th International Symposium on Lameness in Ruminants*, Orlando, Florida, Jan 9-13, 274.

REILLY, J.D., NEWLYN, H., COPE, B., LATHAM, R.J., COLLINS, S. AND HOPEGOOD, L. (2002d) A novel method for assessing hoof horn tubule density (TD) and a comparison of TD in the hooves of ponies, horses, donkeys, cattle, sheep and pigs. *Proceedings of the 12th International Symposium on Lameness in Ruminants*, Orlando, Florida, Jan 9-13, 275.

Appendix 14 has not been copied at the request of the university.

Please contact the university for further information.